ACS chemical biology

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Synthesis and Activity of Thioether-Containing Analogues of the Complement Inhibitor Compstatin

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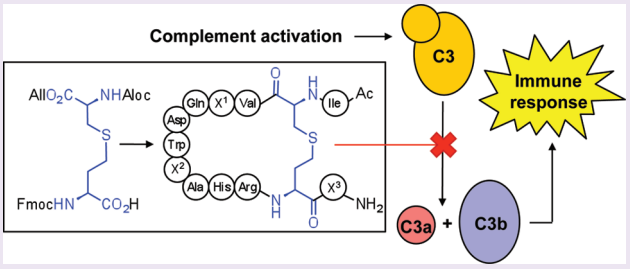
Supporting Information

ABSTRACT: Disulfide bonds are essential for the structural stability and biological activity of many bioactive peptides. However, these bonds are labile to reducing agents, which can limit the therapeutic utility of such peptides. Substitution of a disulfide bond with a reduction-resistant cystathionine bridge is an attractive means of improving stability while imposing minimal structural perturbation to the peptide. We have applied this approach to the therapeutic complement inhibitor compstatin, a disulfide-containing peptide currently in clinical trials for age-related macular degeneration, in an effort to maintain its potent activity while improving its biological stability. Thioether-containing compstatin analogues were produced *via* solid-phase peptide synthesis utilizing orthogonally protected cystathionine amino acid building blocks and solid-supported peptide cyclization. Overall, the affinity of these analogues for their biological target and potent inhibition of complement activation were largely maintained when compared to those of the parent disulfide-containing peptides. Thus, the improved stability to reduction conferred by the thioether bond makes this new class of compstatin peptides a promising alternative for therapeutic applications. Additionally, the versatility of this synthesis allows for exploration of disulfide-to-thioether substitution in a variety of other therapeutic peptides.



Disulfide bonds play a critical role in the structural stability of peptides and proteins.[1] Intricate conformations enforced by cyclic disulfide networks are associated with the potent biological activities of a variety of peptide natural products such as the conotoxins,[2] cyclotides,[3] and defensins.[4] In the case of many bioactive peptides, the structural constraint introduced by disulfide cyclization has been demonstrated to improve resistance to proteolysis and reduce the entropic cost of binding to their targets.[5,6] However, the lability of the disulfide bond to intra- and extracellular reducing agents[7−9] can reduce the biological activity of disulfide-containing peptides and limit their usefulness as therapeutics. To address this concern, substitution of the disulfide bond in bioactive peptides with a variety of different moieties has been explored, including the use of diselenide,[10,11] lactam,[12,13] carba,[14−16] and thioether bridges.[17,18] A disulfide-to-thioether substitution utilizing a cystathionine (Cth) bridge is particularly attractive, since it involves the substitution of only a single atom (-S- to -CH_2-) and results in a carbon−sulfur bond stable to reduction with minimal structural perturbation. Synthetic replacement of disulfides with thioethers has produced analogues of vasopressin,[19] oxytocin,[20] anticardiolipid antibody binders,[21] and VCAM/VLA-4 antagonists.[22] However, as evidenced by lack of recent literature describing Cth-containing peptides, a higher-yielding and more versatile synthetic strategy is needed to allow greater access to these cyclic structures.

Cth is the methylene homologue of lanthionine, found in the lantibiotic family of natural products.[23,24] As such, the considerable body of work on the chemical synthesis of lanthionine-containing cyclic peptides, including lactosin S,[25] bis(desmethyl) lacticin 3147 A2,[26] and fragments of nisin[27,28] and subtilin,[29,30] is directly applicable to the synthesis of Cth analogues of disulfide-bridged peptides. Additionally, the structural constraint associated with cyclic lanthionines has been utilized to improve the activity and/or stability of bioactive peptides, including enkephalin,[18] angiotensin,[31] luteinizing hormone-releasing hormone,[32] and somatostatin.[33] Thus, incorporation of cyclic thioether-containing amino acids is a valuable approach to the development and optimization of therapeutic peptides, and improved methods of constructing such compounds have application to a wide variety of biological problems.

Compstatin (I[CVVQDWGHHRC]T-NH_2) is a 13-residue, disulfide-containing peptide inhibitor of complement activation that was originally discovered *via* screening of a phage-display library.[34] Compstatin inhibits the proteolytic activation of complement component C3 to the anaphylatoxin C3a and the opsonin C3b by C3 convertase complexes (Figure 1a) *via* direct

Received: February 3, 2011
Accepted: April 26, 2011
Published: April 26, 2011

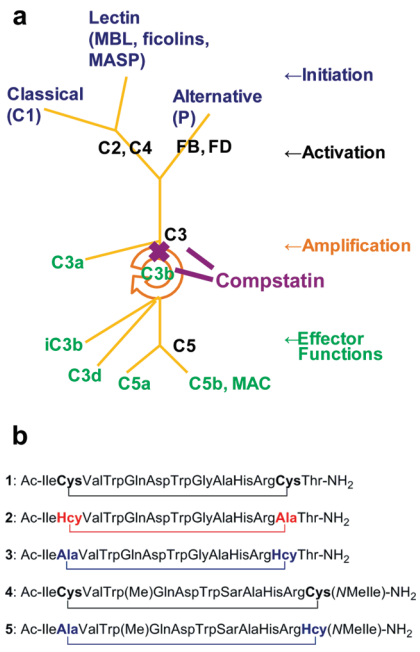
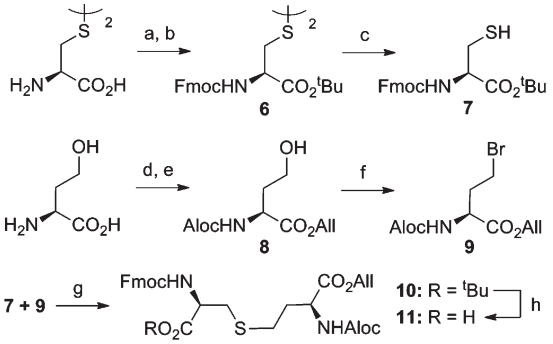


Figure 1. Complement-targeted inhibitory mechanism and peptide sequences of compstatin analogues. (a) The complement cascade is initiated through at least three different mechanisms, which all converge in the proteolytic activation of C3 to C3b; C3b participates in a self-amplified loop that enhances the effect of complement activation. Inhibition by compstatin at the level of C3/C3b effectively stops the activation of all initiation pathways, the amplification loop, and all downstream effects of complement activation. C1−C5: complement components 1−5; FB: factor B; FD: factor D; MAC: membrane attack complex; MBL: mannose-binding lectin; MASP: MBL-associated serine protease; P: properdin. (b) The sequences of compstatin analogues **1**−**5** used in this study. The two cystathionine isomers are depicted as Ala-Hcy or Hcy-Ala, depending on the position of the sulfur atom. Hcy: homocysteine; Trp(Me): 1-methyltryptophan.

binding to C3 as well as C3b.[35−37] The central role of C3 and its proteolytic fragments in all complement initiation and amplification pathways makes compstatin an attractive potential therapeutic for the treatment of pathologies involving inappropriate activation of the complement system,[35,38] including a variety of autoimmune, inflammatory, and neurodegenerative diseases as well as sepsis, hemodialysis-associated thrombosis and transplantation medicine. In particular, compstatin has shown promising activity in treating early forms of age-related macular degeneration and is currently in clinical trials for the treatment of this disease that constitutes a major cause of blindness in the elderly.[39]

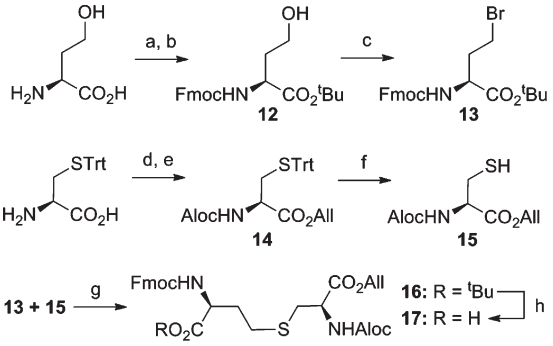
Early work has shown that the activity of compstatin is dependent on disulfide formation between cysteine residues at positions 2 and 12, which enforces the cyclic conformation critical for binding C3.[36] Either reduction−alkylation of the disulfide bond or replacement of the two cysteine residues with alanine results in complete loss of C3 binding and complement inhibition.[34] Furthermore, cyclization protects compstatin from proteolytic degradation in human blood.[40] Extensive structure−activity relationship studies have resulted in derivatives with up to 1000-fold improvement in potency when compared to the original compstatin sequence.[35,41−43] Yet the increasing use of compstatin in both systemic and local (*e.g.*, intravitreal) clinical applications also puts new demands on pharmacokinetic properties such as

Scheme 1. Synthesis of Fmoc-Cystathionine Isomer 11[a]



[a] Reagents and conditions: (a) 70% $HClO_4$, ^tBuOAc; (b) Fmoc-OSu, *N*-methylmorpholine, THF, 62% (two steps); (c) PBu_3, H_2O, THF, 86%; (d) Alloc-Cl, Na_2CO_3, H_2O, CH_3CN; (e) allyl bromide, $NaHCO_3$, DMF, 77% (two steps); (f) PPh_3, CBr_4, CH_2Cl_2, 80%; (g) Bu_4NBr, $NaHCO_3$, H_2O, EtOAc, 84%; (h) CF_3CO_2H, $PhSiH_3$, CH_2Cl_2, 95%.

Scheme 2. Synthesis of Fmoc-Cystathionine Isomer 17[a]



[a] Reagents and conditions: (a) Fmoc-OSu, Na_2CO_3, H_2O, 1,4-dioxane; (b) $CCl_3C(NH)O^tBu$, CH_2Cl_2, THF, 52% (two steps); (c) PPh_3, CBr_4, CH_2Cl_2, 94%; (d) Alloc-Cl, Na_2CO_3, H_2O, CH_3CN; (e) allyl bromide, $NaHCO_3$, DMF, 70% (two steps); (f) CF_3CO_2H, $^iPr_3SiH$, CH_2Cl_2, 85%; (g) Bu_4NBr, $NaHCO_3$, H_2O, EtOAc, 93%; (h) CF_3CO_2H, $PhSiH_3$, CH_2Cl_2, 98%.

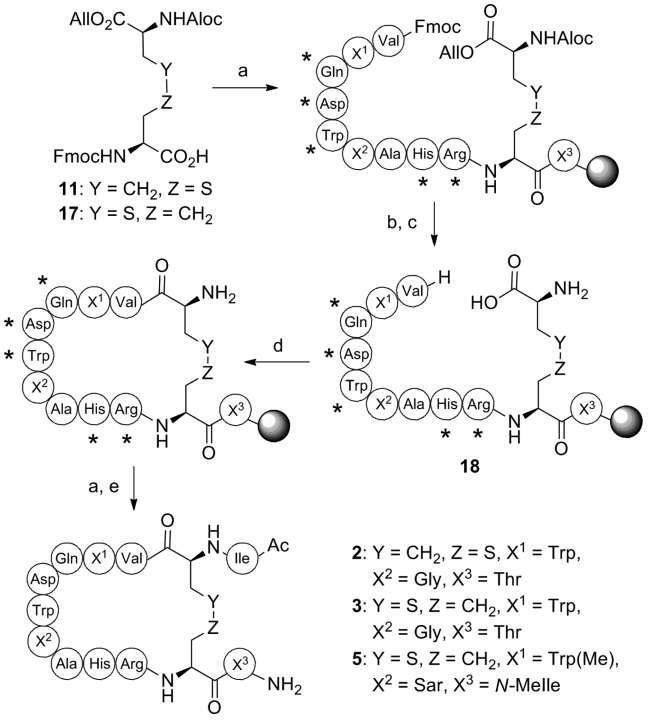
metabolic stability. Here, we report the synthesis of a series of compstatin analogues **2**, **3**, and **5** (Figure 1b) containing a reduction-resistant Cth bridge in place of disulfide, which maintain potent complement inhibitory activity and strong affinity for C3b.

RESULTS AND DISCUSSION

Cth Building Block Synthesis. We desired a versatile and high-yielding synthetic strategy to produce a family of Cth-containing compstatin analogues, which could also be applied to the synthesis of other Cth-containing peptides. In this regard, we adopted a 9*H*-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) that was modified from the approach used by Vederas and co-workers for the total synthesis of lantibiotics.[25,26] This strategy involved the synthesis of two enantiomerically pure, orthogonally protected LL-Cth building blocks, representing the two possible sulfur-to-methylene substitutions from the parent disulfide, followed by solid-supported peptide assembly and cyclization. This building block approach allows for complete positional freedom for the placement of the Cth bridge within the cyclic peptide. Construction of Cth building

dx.doi.org/10.1021/cb2000378 |*ACS Chem. Biol.* 2011, 6, 753–760

Scheme 3. Synthesis of Cystathionine-Containing Compstatin Analogues 2, 3, and 5[a]



[a] Reagents and conditions: (a) SPPS; (b) $Pd(PPh_3)_4$, $PhSiH_3$, DMF; (c) piperidine, DMF; (d) PyBOP, HOAt, $^iPr_2EtN$, DMF; (e) CF_3CO_2H, H_2O, $^iPr_3SiH$, $HS(CH_2)_2SH$. An asterisk indicates standard Fmoc-orthogonal side chain protection: Gln(Trt), Asp(O^tBu), Trp(Boc), His(Trt), Arg(Pbf), Thr(^tBu).

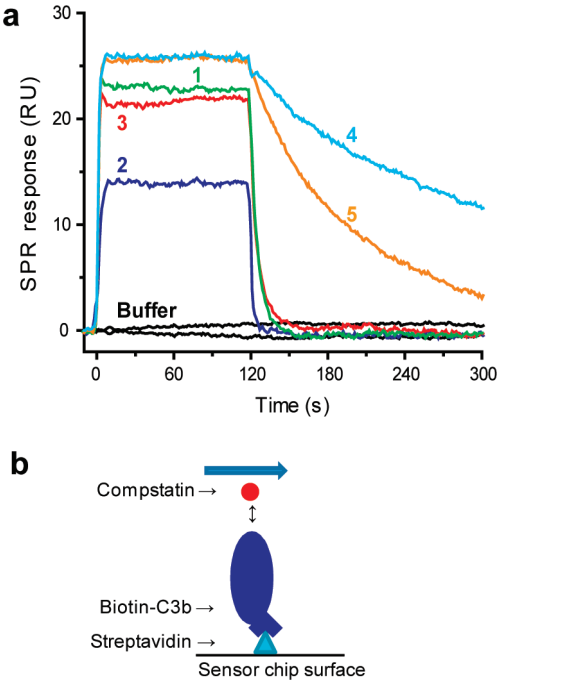


Figure 2. Kinetic ranking of compstatin analogues. (a) Analogues 1-5 at 1 μM concentration were injected over captured C3b at a surface density of 3000 RU. The signals are overlaid to show relative differences in their association and dissociation phases. Each plot is representative of three data sets. RU: resonance units. (b) Schematic representation of the SPR assay.

block **11** (Scheme 1) proceeded in high yield *via* a convergent synthesis. Phase-transfer condensation[44] of Fmoc- and *tert*-butyl ester-protected L-cysteine **7**, derived from L-cystine, with allyloxycarbonyl (Aloc)- and allyl ester (OAll)-protected γ-bromo-L-aminobutyrate **9**, derived from L-homoserine, yielded the protected Cth building block **10**. The *tert*-butyl ester of **10** was subsequently removed to reveal the free carboxyl group in **11** necessary for SPPS. Synthesis of the other Cth isomer **17** (Scheme 2) was similarly performed *via* phase-transfer condensation of γ-bromoaminobutyrate derivative **13** with cysteine derivative **15**.

The stereochemical purity and absolute confirmation of the synthesized LL-Cth building blocks was assessed *via* chiral gas chromatography/mass spectrometry (GC/MS) using a Varian CP-Chirasil-L-Val column (Supporting Information). Compound **17** was globally deprotected and derivatized as the pentafluoropropionamide methyl ester.[25,45] Comparison of this compound with derivatized standards of LL- and DL-cystathionine confirmed the expected LL-configuration of the synthetic Cth building block with no detectable epimerization of the enantiomerically pure starting materials during synthesis.

Peptide Synthesis. Three Cth-containing compstatin analogues (**2**, **3**, and **5**) were prepared. Peptides **2** and **3** represent the two possible Cth analogues of the second-generation compstatin derivative 4W9A (control peptide **1**, Figure 1b),[36] with the Cth-based thioether bridge replacing the original Cys2−Cys12 disulfide bond. Our initial assessment of binding data with peptides **1**−**3** (see Kinetic Analyses below) indicated that the location of the sulfur atom closer to the *N*-terminus, as in **3** that results from building block **17**, yields a more potent complement inhibitor. Based on those results, we used **17** in the synthesis of analogue **5**, which includes three additional mutations (Trp^4/Trp(Me), Gly^8/Sar, and Thr^{13}/*N*-MeIle) found in the most active compstatin sequence reported to date, Compstatin 20 (control peptide **4**, Figure 1b).[41]

Construction of compstatin analogues **2**, **3**, and **5** (Scheme 3) utilized Fmoc-based SPPS on Rink amide AM resin and 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) as the coupling reagent. As reported for the synthesis of the lanthionine-containing peptide bis-(desmethyl)lacticin 3147 A2,[26] intermolecular coupling between the distal ends of two resin-bound lanthionine residues becomes problematic during cyclization when using resin loadings greater than 0.3 mmol/g. Therefore, in order to promote the desired intramolecular cyclization, resin loading was reduced to 0.2 mmol/g by coupling a substoichiometric amount of the Cth building block, followed by acetylation of the remaining free resin sites with acetic anhydride (Methods). After coupling of the remaining residues inside the incipient cystathionine ring, the allyl protecting groups of the Cth residue were removed with $Pd(PPh_3)_4$ and $PhSiH_3$, and the *N*-terminal Fmoc group was removed with piperidine. Cyclization of this deprotected resin-bound fragment **18** was promoted with (benzotriazole-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxy-7-azabenzotriazole (HOAt), and diisopropylethylamine. Coupling of the *N*-terminal isoleucine, Fmoc removal, amine acetylation, and trifluoroacetic acid-promoted global deprotection/resin cleavage yielded the crude compstatin analogues, which were purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The pure peptides were

dx.doi.org/10.1021/cb2000378 |*ACS Chem. Biol.* 2011, 6, 753–760

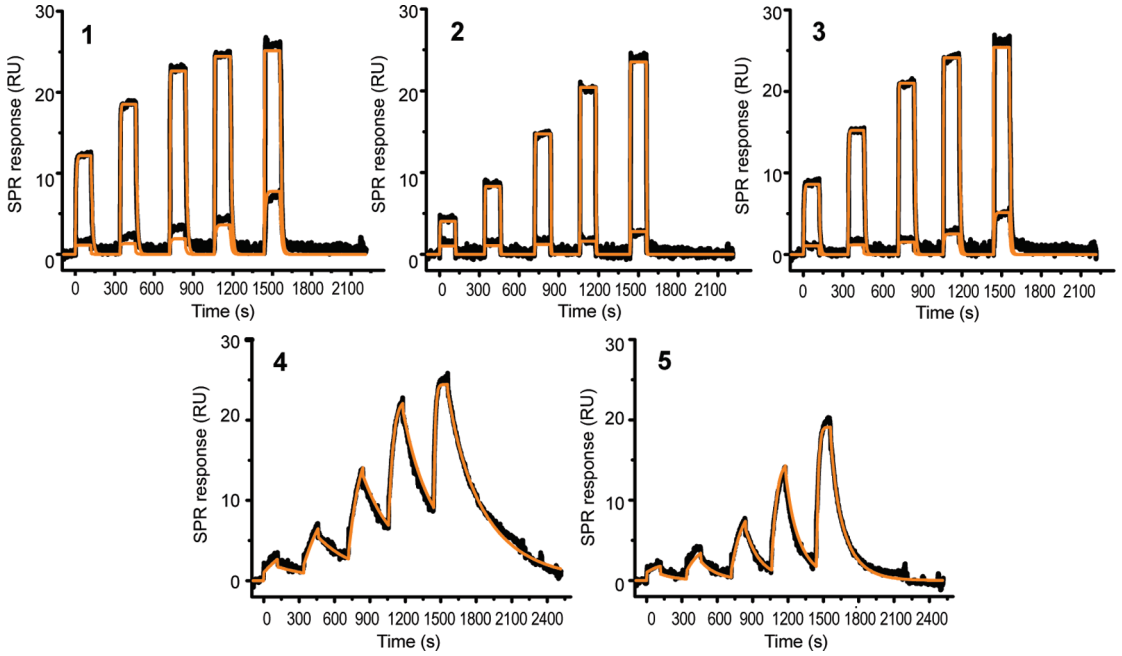


Figure 3. Single-cycle kinetics analysis of the thioether compstatin analogues and corresponding disulfide bond controls. Sets of five increasing concentrations were consecutively injected over a C3b surface (3000 RU density) in a single cycle. In the case of peptides **1**−**3**, two sets of concentrations are superimposed for a better description of the full binding range and to achieve a well-defined affinity profile. The processed signals were fitted to a 1:1 binding model (orange simulation curves) and kinetic rate constants k_a and k_d were extracted (Table 1). The compstatin analogue used is shown in the top left corner of each plot. Plots are representative of at least six data sets.

obtained with average overall yields of 2.4−5.0%, or 88−90% per step over 29 steps. Peptide **5** had the lowest overall yield, presumably due to difficulty in coupling the Cth building block immediately after a sterically demanding *N*-methylisoleucine residue.

Chiral GC/MS was again utilized in order to confirm that the LL-stereochemistry of the Cth building blocks was maintained during compstatin analogue synthesis (Supporting Information). Peptide **5** was hydrolyzed in refluxing 6 M HCl, and the resulting amino acids were derivatized as the pentafluoropropionamide methyl esters and compared to the derivatized LL- and DL-cystathionine standards as described above. The vast majority of the derivatized cystathionine hydrolyzed from **5** contained the appropriate LL-configuration, confirming that the Cth building blocks are configurationally stable to the basic conditions employed during SPPS.

Kinetic Analyses. The resulting Cth-containing compstatin analogues were then assayed to determine their affinity for C3b and their effectiveness in inhibiting complement activation. Kinetic characterization of the interaction of each compstatin analogue with C3b was performed using surface plasmon resonance (SPR). Biotinylated C3b was site-specifically captured on a streptavidin-coated sensor chip to prevent surface heterogeneity.[38,42] A kinetic ranking of the compstatin analogues at 1 μM (Figure 2) indicated that the association and dissociation profiles of peptide **3** were the most comparable to those of its corresponding control peptide **1**. Peptide **2**, on the other hand, displayed a significantly lower binding activity compared to **1**. In the case of Compstatin 20, analogue **5** showed a similar association but faster dissociation rate than the corresponding control peptide **4**.

To further characterize and quantitate the relationship between the structure and activity of these compounds, we performed a full kinetic analysis. The slow dissociation rate constant of the current lead compstatin sequence **4**[41] would require a long assay time to allow the signal to reach baseline level before the subsequent injection or the use of regeneration solutions that remove residual peptide yet may harm the activity of C3b. We therefore opted for a single-cycle kinetics approach,[46] which allows for kinetic characterization of interactions with slow dissociation rates in a shorter period of time than does the traditional multicycle approach.[40] This approach was used for all peptides, so as to enable us to make direct comparisons of the acquired results. Importantly, all compounds showed a binding response that could be fitted to a 1:1 binding model. The single-cycle kinetics analysis showed fast association and dissociation rates for compounds **1**, **2**, and **3**, and fast association but slower dissociation for compounds **4** and **5** (Figure 3, Table 1). The compounds mainly differ in their dissociation rates, whereas the association rate remained rather constant within the corresponding series of analogues. The resulting binding affinity is therefore mainly defined by the changes in k_d. In particular, peptide **2** displayed a 6-fold faster dissociation and a 6-fold reduced affinity

Table 1. Kinetic Rate Constants, Binding Affinities, Inhibitory Potential, and Relative Inhibitory Activities (rIC_{50}) of Compstatin Analogues

peptide	k_a (10^5 M^{-1} s^{-1})[a]	k_d (10^{-2} s^{-1})[a]	K_D (nM)[a]	IC_{50} (nM)[b]	rIC_{50}
1	5.6 ± 1.4	8.1 ± 1.1	150 ± 19	1900 ± 1200	1
2	5.5 ± 0.7	46.8 ± 5.7	847 ± 59	>20000	>10
3	4.6 ± 0.5	11 ± 1.9	236 ± 20	3100 ± 1800	1.6
4	18.6 ± 4.1	0.4 ± 0.1	2.4 ± 0.6	130 ± 40	1
5	16.4 ± 3.8	1.5 ± 0.4	9 ± 1.8	270 ± 80	2.1

[a] Results show average and standard deviations of at least six data sets. [b] Results show average and standard deviations of at least five data sets.

dx.doi.org/10.1021/cb2000378 |*ACS Chem. Biol.* 2011, 6, 753–760

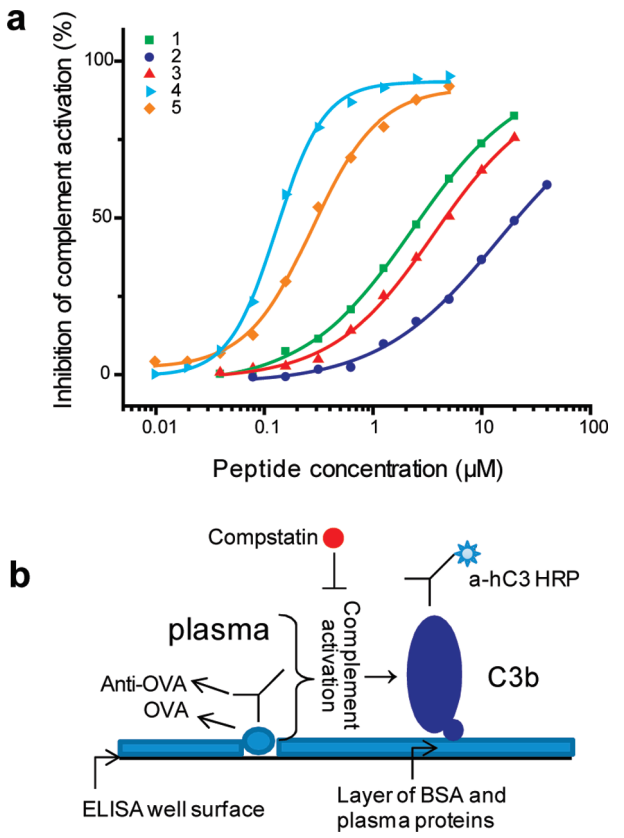


Figure 4. Inhibitory activity of compstatin analogues. (a) Representative assay result for inhibition of antigen/antibody complex-initiated complement activation by compstatin analogues. The percentage inhibition is plotted against the peptide concentration and compared to that for parent compstatin analogues **1** and **4**. Plots are representative of at least five assays. BSA: bovine serum albumin; OVA: ovalbumin; a-hC3 HRP: anti-human C3 antibody conjugated to horseradish peroxidase. (b) Schematic representation of the assay.

when compared to control peptide **1**. Peptide **3**, on the other hand, had a slightly slower association rate (1.2-fold) and slightly faster dissociation rate (1.4-fold), yielding a ~1.6-fold lower affinity for C3b when compared to peptide **1**. Finally, compound **5** displayed a comparable association rate but a ~3-fold faster dissociation rate than the corresponding control peptide **4** and thus a ~3.8-fold lower affinity for C3b. Importantly, the kinetic parameters for the control peptides **1** and **4** were very similar to those published previously.[40,41] The largely stable association rate constants within each compound series suggest that the cystathionine modification had little effect on complex formation with C3b and appears to maintain the ability of compstatin peptides to change between the distinct solution and bound conformers.[36,41] However, the slightly changed geometry between a disulfide and a thioether bridge may have influenced the exact positioning of key residues such as the tryptophan derivatives at position 4 and 7, thereby affecting the complex stability as measured by k_d. This effect appears more pronounced in **5**, as the mutations made in control peptide **4** better tailored the peptide to the binding site. Importantly, our kinetic data clearly show that thioether-bridged analogues of compstatin show high binding affinities in the range of the compound currently in clinical trials (1Me-Trp;[4] K_D = 11 nM).[42]

Complement Inhibition Assays. The biological activity of the Cth-containing compounds was also compared to that of their corresponding disulfide-containing control peptides (Figure 4, Table 1). The ability of these compounds to inhibit antigen/antibody-induced complement activation was assessed by measuring the resulting reduction in C3b deposition on an ELISA plate when compared to a control well with no peptide. The percentage inhibition was then plotted against the peptide concentrations and fitted to a logistic dose−response function. These assays yielded results consistent with the data obtained in the kinetic assays. Again, peptide **2** was identified as the least active analogue, thereby confirming the unfavorable effect of sulfur-to-methylene substitution on the *C*-terminal side of the original disulfide. In both series, the thioether-containing peptides **3** and **5** showed a decreased inhibitory activity by a factor of only 2 when compared to their parent disulfide-linked peptides **1** and **4**, respectively (Table 1). In agreement with previous studies, these results show a good correlation between the binding affinity for C3b and the ability to inhibit complement activation. Importantly, the combined analyses also confirm thioether-restricted compstatin analogues as potent complement inhibitors.

Effect of Oxidation and Reduction on Peptide Activity. The resistance of the cyclic structure of the Cth-containing peptide **5** to reductive treatment and, conversely, the reductive lability of the corresponding disulfide-linked analogue **4** was confirmed by incubating the peptides with 500-fold molar excess of the reducing agent tris(2-carboxyethyl)-phosphine hydrochloride (TCEP-HCl) and subsequent analysis by mass spectrometry (Supporting Information). In order to confirm that recent compstatin analogues with largely increased potency and affinity still depend on an intact ring structure, we subjected peptide **4** after reduction and alkylation to binding and complement inhibition analysis as described above. This analysis is necessary in the context that analogues containing sarcosine instead of glycine at position 8 (including peptides **4** and **5**) have been implied to form a bound-like conformation in solution[41] that may have rendered maintenance of the disulfide less important. However, consistent with previous analogues,[40] peptide **4** was rendered essentially inactive by the reduction process (Supporting Information), thereby confirming the importance both of the cyclic structure and of reductive stability. Whereas cyclic, *N*-acetylated compstatin analogues have previously been shown to exert high plasma stability under normal conditions,[40] there are a variety of complement-related diseases that trigger changes in the reductive status of blood. For example, compstatin has shown promise as an organ-protecting agent in models of bacterial sepsis,[47] but this pathology also involves a large increase in the plasma levels of thioredoxin that may endanger the disulfide structure.[48] Similarly, complement inhibition by compstatin has large potential in hemolytic conditions such as paroxysmal nocturnal hemoglobinuria [38] or hemodialysis-induced side effects,[49] during which the large reservoir of reduced glutathione (GSH) in erythrocytes (1−3 mM) is released and increases the plasma pool of GSH.[50,51] In these compstatin-relevant therapeutic applications, protection of the peptide against reduction may therefore become even more important.

Although thioethers are inert to reductive cleavage, their lability to metabolic oxidation is well documented, especially in the case of methionine in peptide and protein drugs.[52,53] In addition, thioether oxidation has been shown to severely decrease the activity of the lantibiotic nisin,[54] although the lantibiotic actagardine contains an enzymatically oxidized methyllanthionine bridge important for full activity.[55] In our hands, the Cth-containing building blocks and peptides appear stable to spontaneous oxidation

dx.doi.org/10.1021/cb2000378 |*ACS Chem. Biol.* 2011, 6, 753–760

during synthesis, purification, biological testing, and storage. However, to probe the effect of thioether oxidation on peptide binding and inhibitory activities, we exposed peptides **4** and **5** to conditions that allow selective oxidation of Cth to the corresponding sulfoxide by hydrogen peroxide (Supporting Information). Oxidation of peptide **5** led to a mass shift of +16 Da, whereas peptide **4** was largely unaffected by the treatment. As expected, the oxidative treatment of peptide **4** did not significantly affect its inhibitory potency (ΔIC_{50} < factor 2). Although Cth-oxidation of peptide **5** did induce some loss in both binding affinity and inhibition activity (4−6-fold), the oxidized peptide was still a highly potent inhibitor with a K_D below 100 nM (Supporting Information).

Conclusion. We describe here the chemical synthesis of analogues of the complement inhibitor compstatin with a reduction-stable thioether bond in place of the reduction-labile disulfide bond. This modification was installed *via* a versatile and high-yielding solid-phase synthesis utilizing orthogonally protected cystathionine building blocks. Furthermore, we have characterized the structure −activity relationships of these cystathionine-containing analogues and the disulfide-bonded parent peptides. Our results indicate that the position of the sulfur atom within the thioether bridge has consequences for activity, as δ-Cth-containing analogues largely maintained the binding and inhibitory properties of the disulfide parent, but γ-Cth-containing analogues did not. In comparing potential metabolic alterations of these compounds, reduction of the disulfide bond had a much more deleterious effect on potency than potential oxidation of the cystathionine bridge, thereby underscoring the benefit of the substitution in this context. More generally, the impact of peptide-based drugs continues to grow, and many of these compounds contain disulfide bonds.[56,57] The versatile nature of the synthesis described here allows for the production of a wide variety of cyclic Cth-containing peptides in order to probe the effects of disulfide substitution on the activity and stability of other therapeutic peptides.

METHODS

Synthesis of Peptides 2, 3, and 5 *via* SPPS. Fmoc-protected Rink amide AM resin (0.6 mmol/g loading) was swelled in dimethylformamide (DMF) for 20 min and Fmoc-deprotected with 20% piperidine in DMF (2 × 10 min). The first amino acid (Fmoc-Thr-OH for **2** and **3**, Fmoc-*N*-Me-Ile-OH for **5**; 4 equiv to resin) was preactivated in the presence of HCTU (4 equiv) and diisopropylethylamine (8 equiv) in DMF for 5 min and then coupled to the resin for 1 h *via* agitation with N_2. The Fmoc group was then removed. In order to prevent interstrand dimerization upon attempted cyclization, the resin loading was manually reduced to 0.15 mmol/g by reacting a preactivated solution of **11** or **17** (0.25 equiv to resin), HCTU (0.25 equiv), and diisopropylethylamine (2 equiv) in DMF with the resin-bound peptide for 3 h. The remaining free resin sites were acetylated with a solution of 1:1:3 acetic anhydride/diisopropylethylamine/DMF for 30 min, yielding a negative Kaiser test.

After resin-loading modification, the resin-bound peptide was Fmoc-deprotected with 20% piperidine in DMF (2 × 10 min), the reaction vessel was drained, and the resin was washed with DMF and CH_2Cl_2. Appropriately side chain-protected Fmoc-amino acids (4 equiv with respect to the reduced-loading resin) were preactivated in the presence of HCTU (4 equiv) and diisopropylethylamine (8 equiv) in DMF for 5 min and then coupled to the deprotected resin for 45−60 min. The reaction vessel was drained, and the resin was washed with DMF and CH_2Cl_2. A negative Kaiser test confirmed completed couplings.

Following the coupling of Fmoc-Val-OH to the resin-bound peptide, a solution of $Pd(PPh_3)_4$ (1 equiv to the reduced-loading resin) and $PhSiH_3$ (10 equiv) in 1:1 DMF/CH_2Cl_2 was added to the resin and reacted for 2 h, protected from light. The reaction vessel was drained, and the resin was washed with 0.5% diethyldithiocarbamate in DMF, DMF, and CH_2Cl_2. The Fmoc group was removed with 20% piperidine in DMF (2 × 10 min). Peptide cyclization was performed by treating the resin-bound linear peptide with PyBOP (5 equiv), HOAt (5 equiv), and diisopropylethylamine (10 equiv) in DMF for 2 × 1.5 h. The *N*-terminal Fmoc-Ile-OH was then coupled, deprotected, and acetylated as described above.

The resin was washed with DMF and CH_2Cl_2 and then dried. Concurrent side-chain deprotection and cleavage from resin were achieved by stirring the resin-bound peptide in 91.5:5:2.5:1 trifluoroacetic acid/water/triisopropylsilane/ethanedithiol for 2 h under N_2. The cleaved resin was removed by filtration, and the filtrate was concentrated by a stream of N_2. Crude peptide was precipitated with the addition of cold Et_2O, isolated, and lyophilized from 1:1 water/acetonitrile. The dry crude peptide was purified by RP-HPLC employing the gradients described below (solvent A is 0.1% trifluoroacetic acid in water; solvent B is 80% acetonitrile in water with 0.086% trifluoroacetic acid). Product-containing fractions were pooled, lyophilized, and checked for identity by MALDI-TOF MS.

Peptide 2. This compound was purified on a Waters Delta-Pak C18 preparative column with the following gradient: 2% solvent B for 1 min, 2−20% B over 2 min, 20−26% B over 3 min, 26−41% B over 30 min. After lyophilization, 8 mg of pure peptide was obtained from a 0.1-mmol-scale synthesis (5.0% total yield, 90% per step over 29 steps). t_R = 31.9−32.8 min. LRMS (MALDI-TOF) calcd for $C_{72}H_{103}N_{22}O_{18}S$ 1595.7, found 1595.7.

Peptide 3. This compound was purified in the same fashion as **2**. After lyophilization, 6 mg of pure peptide was obtained from a 0.1-mmol-scale synthesis (3.8% total yield, 89% per step over 29 steps). t_R = 31.8−32.8 min. LRMS (MALDI-TOF) calcd for $C_{72}H_{103}N_{22}O_{18}S$ 1595.7, found 1596.0.

Peptide 5. This compound was purified on a Phenomenex Luna C18 preparatory column with the following gradient: 5% B for 1 min, 5−24% B over 2 min, 24−30% B over 3 min, 30−45% B over 30 min. After lyophilization, 12 mg of pure peptide was obtained from a 0.3-mmol-scale synthesis (2.4% total yield, 88% per step over 29 steps). t_R = 26.7−27.8 min. LRMS (MALDI-TOF) calcd for $C_{77}H_{113}N_{22}O_{17}S$ 1649.8, found 1649.9.

Synthesis of Control Peptides 1 and 4. The synthesis and characterization of these peptides has been described previously.[41,58]

Kinetic Analyses. C3 was purified from human plasma as described before.[43] C3b was generated by limited trypsinization and labeled with biotin-maleimide using established protocols.[59] The interaction of the compstatin analogues with C3b was characterized using a Biacore 3000 instrument (GE Healthcare, Corp., Piscataway, NJ) at 25 °C. The running buffer was PBS, pH 7.4 (10 mM sodium phosphate, 150 mM NaCl) with 0.005% Tween-20. Biotinylated C3b was captured site-specifically on a streptavidin chip at ∼3000, 4000, and 5000 RU density; an untreated flow cell was used as a reference surface. An initial kinetic ranking was performed by injecting the various peptides at a constant concentration of 1 μM for 2 min. For detailed kinetic analysis, sets of five increasing concentrations of a particular compound were consecutively injected over the chip surface in a single cycle without waiting for full dissociation between injections (kinetic titration).[46] A 3-fold dilution series (one set at 0.46−37 nM for analogues **4** and **5**, and two sets at 0.46−37 and 111−9000 nM for all other analogues) was injected at a flow rate of 30 μL/min; each injection was done for 2 min, allowing the peptide to dissociate for 5 min each time before the next injection was started. After the end of the last injection, a 10-min dissociation was used for compounds **1**, **2** and **3**, while a 15-min dissociation was used for compounds **4** and **5**. Data processing and analysis was performed using Scrubber (BioLogic Software, Campbell, Australia) and BIAevaluation (GE Healthcare). A series of blank injections was subtracted from the binding signals

(double referencing) and the processed signals were fitted to a single cycle 1:1 binding model (kindly provided by GE Healthcare) to extract the kinetic rate constants (k_a, k_d) and calculate the binding affinity ($K_D = k_d/k_a$).

Complement Inhibition Assays. To assess the complement inhibitory ability of the compstatin analogues, an enzyme-linked immunosorbent assay (ELISA) was performed as previously described[60] with small modifications. In brief, an ovalbumin-based antigen/antibody complex was used as an initiator of the classical pathway of complement activation. Serial dilutions of the compstatin analogues were prepared, and normal human plasma was added to a final dilution of 1:80 in veronal-buffered saline containing $MgCl_2$ and $CaCl_2$. Incubation for 15 min followed to allow complement activation and amplification. The deposition of C3b on the plate was measured with a polyclonal antibody against C3. The percentage of inhibition was plotted against the compstatin concentration and fitted to a logistic dose−response function using Origin (OriginLab Corp., Northampton, MA).

■ ASSOCIATED CONTENT

Supporting Information. General materials and methods, synthetic procedures and characterization for synthetic compounds **6-17**, effects of oxidation/reduction on the activity of peptides **4** and **5**, and GC/MS procedures and chromatograms. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

We thank Dr. Deborah McClellan for editorial assistance. This work was supported by the Robert C. and Carolyn J. Springborn Endowment (P.J.K.), the Howard Hughes Medical Institute (W.A.V.). and National Institutes of Health grants T32 GM070421 (P.J.K.), GM062134 (J.D.L.), and AI068730 (J.D.L.).

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