Development of a Peptidomimetic Ligand for Efficient Isolation and Purification of Factor VIII via Affinity Chromatography

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Received March 16, 2007

Hemophilia A, one of the most severe bleeding disorders, results from an inherited deficiency of factor VIII (FVIII) function. Treatment by injection of FVIII has been a common procedure for decades. Nevertheless, the production and purification of FVIII remains a challenging task. Current procedures using immunoaffinity chromatography are expensive and suffer from the instability of the applied antibody ligands, which elute along with the product and contaminate it. Recently, FVIII was purified by use of octapeptide ligands, but their low protease-resistance limits their application. We here report the systematic rational and combinatorial optimization procedure that allowed us to transfer the octapeptide ligands into a small peptidomimetic. This compound is the smallest ligand known for separation of such a large protein (330 kDa). It not only binds and purifies FVIII with high efficiency but also is stable, protease-resistant, and cheap to produce in preparative scale. Hence it offers a valuable alternative to antibody-based purification procedures.

Introduction

Hemophilia A is one of the most common and severe bleeding disorders,[1] affecting approximately one in 5000 males worldwide.[1,2] The severe form of the disease is characterized by spontaneous bleedings, as well as uncontrollable bleedings in case of trauma or surgery. Other clinical hallmarks are acute recurrent painful hemarthroses, which can progress to chronic arthropathy characterized by progressive destruction of the cartilage and the adjacent bone, muscle hematomas, intracerebral hemorrhages, and hematuria.[3] The disease is the result of an X-linked inherited deficiency of coagulation factor VIII (FVIII)[a] function.[2,4,5] FVIII is an essential component of the intrinsic blood coagulation pathway, an intricate order of a variety of enzymatic reactions.[2,6,7] The current treatment for hemophilia A is the infusion of FVIII, either purified from human blood plasma or expressed in recombinant cells,[8−11] which normalizes the clotting process and stops or prevents bleeding.

The FVIII molecule (∼330 kDa, 2332 amino acid residues) is a secretory glycoprotein consisting of three homologous A domains, two homologous C domains and the unique B domain, which are arranged in the order A1-A2-B-A3-C1-C2.[12,13] The protein circulates in blood in complex with von Willebrand factor (vWf), which protects and stabilizes it.[14−18] The large B domain is not important for the FVIII procoagulant activity,[19] but recent studies demonstrated its role in protecting the activated form of FVIII from proteolytic inactivation.[20] Prior to its secretion into plasma, FVIII is processed intracellularly to a series of noncovalently associated, metal(II)-linked heterodimers by cleavage at the B−A3 junction.[7,21,22] The cleavage generates the heavy chain (HCh) consisting of the A1, A2, and B domains and the light chain (LCh) composed of the A3, C1, and C2 domains.[1,7] The resulting protein varies in size due to additional cleavages within the B domain, giving molecules with different lengths.[12,23] Thus, preparations of plasma-derived human FVIII (pdFVIII) contain a heterogeneous mixture of differently sized FVIII molecules.

The survival and well-being of people with hemophilia depends on the supply of safe therapeutic products.[24] In the past, numerous hemophilia patients have been infected with human immunodeficiency virus 1 (HIV-1) or hepatitis C virus via injections of contaminated plasma-derived FVIII preparations.[1,8,25] While the safety of pdFVIII products has been continuously improved during the last decades,[8,26,27] the isolation of the factor VIII gene in 1984 opened new opportunities for treatment.[28−30] The preparation of novel recombinant FVIII (rFVIII) molecules significantly improved supply and product safety.[10,31−34] Nevertheless, rFVIII is a biologically derived product produced by cell culture and carries a risk of transmitting infectious agents.[35] Approximately 25% of the first-generation rFVIII concentrates were positive for transfusion-transmitted viruses from contaminated human serum albumin, added as stabilizer.[36] In contrast, second-generation full-length rFVIII (FL-rFVIII) products as well as the first licensed B-domain-deleted recombinant factor VIII (BDD-rFVIII) molecule[37] do not have added albumin and instead use sucrose or other non-human-derived material as a stabilizer.[10] This advancement significantly improved the product safety and the therapeutic use of recombinant FVIII has been significantly increased in recent years.[38]

However, so far human albumin is still added to the cell culture media and also the use of monoclonal antibodies (mAbs)

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[a] Abbreviations: 1-Nal, 1-naphthylalanine; 3-IAA, 3-indolylacetic acid; 3-IBA, 3-indolylbutyric acid; 3-IPA, 3-indolylpropionic acid; 4-py, 4-pyridylalanine; Ahx, 6-aminohexanoic acid; BDD-rFVIII, B-domain-deleted recombinant factor VIII; Boc, *tert*-butyloxycarbonyl; DIPEA, *N*,*N*-diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMEM, Dulbecco's modification of Eagle's medium; FBS, fetal bovine serum; FL-rFVIII, full-length recombinant factor VIII; Fmoc, 9-fluorenylmethyloxycarbonyl; FVIII, factor VIII; HCh, heavy chain; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; LCh, light chain; mAb, monoclonal antibody; MMP-3, matrix metalloproteinase 3; pdFVIII, plasma-derived FVIII; rFVIII, recombinant FVIII; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TCP, tritylchloride polystyrol; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; Trt, trityl; vWf, von Willebrand factor.

10.1021/jm070304x CCC: $37.00
Published on Web 08/11/2007

as ligands in affinity purification holds a risk of transfusing pathogens. Especially transmissible spongiform encephalopathies and new variants of Creutzfeldt−Jakob disease due to prions as well as previously unknown pathogens, including new murine viruses, may contaminate today's rFVIII products.[35,39] Hence, all efforts should be made to eliminate all proteins from human or animal origin from the manufacturing process of recombinant products.[40] Consequently there is a great demand for novel procedures avoiding such components in order to develop the next generation of recombinant FVIII products.

Besides product safety, financial aspects are major factors in the development of new FVIII products. FVIII therapy is quite expensive, costing approximately $100 000 (U.S.) per year for one patient.[34] As a result, 88% of the safer but more expensive rFVIII is consumed in Europe and North America, while the majority of the world's population with hemophilia is still reliant on blood products or does not receive any treatment at all due to economic reasons.[8,9,24,25,41] Novel techniques reducing production costs can help to improve the FVIII supply and make treatment accessible for a broader range of the population.

The purification of FVIII remains a challenging task. It involves a complex sequence of different purification techniques such as affinity chromatography,[42] ion-exchange chromatography, and virus inactivation.[37,43−49] Today, all recombinant FVIII preparations and many plasma-derived FVIII products are purified via immunoaffinity chromatography employing mAbs as ligands.[10,50,51] Nevertheless, the use of protein ligands in affinity chromatography is not only very expensive but also limited by several other factors:[52−55] Antibodies are known to be eluted along with the product, contaminating or inactivating it or even evoking immune responses.[43,47,55] As biologically derived products, they show lot-to-lot variation and they may be contaminated with, for example, host DNA, viruses, or prions that can be transfused to the product.[54] Their low stability shortens the column life and they suffer from low binding capacities, limited life cycles, and low scale-up potential.[52,54,55] Moreover, because of the very strong binding of antibody ligands to FVIII, harsh conditions are required for elution of the protein, which can harm both the target protein and the ligand.

Synthetic ligands like peptides have had so far only limited use in affinity separation. However, the introduction of combinatorial libraries has expanded the repertoire of affinity ligands for such compounds.[56] Two independent groups recently reported the development of oligo- and polypeptides as affinity ligands for factor VIII. Kelley et al.[57,58] described the isolation of a 27 amino acid sequence using phage display techniques. The cyclic polypeptide is currently used in the manufacturing of a third-generation BDD-rFVIII product, currently in clinical trials.[48] Another promising result was reported by the group of Jungbauer.[59,60] They found a series of octapeptides with high affinity toward FVIII derived from a combinatorial library using spot technology on cellulose sheets. The immobilized ligands could be used to purify FVIII from diluted plasma.

Nevertheless, the application of oligo- and polypeptides is associated with several problems restricting their use, above all their high susceptibility toward enzymatic degradation.[52,55] This significantly limits their application, as raw materials such as serum or cell culture extracts contain proteases. Degradation of the ligands, covalently bound to the column medium, may rapidly lead to inefficiency and reduced selectivity and consequently shorten the life of the expensive affinity column. Furthermore, peptides, although cheaper than antibodies, are still quite cost-intensive and not trivial to produce in large scale, as they are typically synthesized on solid phase and purified by HPLC.

Even so, peptidic ligands are promising lead structures for the development of small unnatural molecules. Such compounds have the potential to reduce production costs and to improve the safety of current and future FVIII products. Here we present the systematic downsizing of an octapeptidic FVIII ligand into a small peptidomimetic ligand. This novel ligand is protease-resistant and binds FVIII with high affinity. Using this ligand, we were able to isolate FVIII of high purity from a complex mixture containing contaminant proteins in vast excess. Moreover, a stereoselective straightforward solution synthesis was developed, allowing cost-effective production of the ligand on a preparative scale.

Table 1. Optimization of Ligand **7**: Loading Density and Absolute FVIII Binding Data for Various Mutants of Ligand **7**

sequence	no.	ligand loading (μmol/mL)	^{125}I-pdFVIII binding[a] (%)
YCSWEY	**7**	17.0 ± 0.5	62.0 ± 0.40
YCSWDY	**8**	16.7 ± 1.0	69.8 ± 12.2
YCS(1-Nal)EY	**9**	12.7 ± 0.6	35.3 ± 3.00
YCAWEY	**10**	12.2 ± 0.6	72.1 ± 7.20
YCTWEY	**11**	14.9 ± 0.7	70.7 ± 2.40
YCVWEY	**12**	12.9 ± 0.6	94.1 ± 4.70
YCAWDY	**13**	19.3 ± 1.0	72.0 ± 15.5
YCTWDY	**14**	17.6 ± 0.0	73.6 ± 0.70
YCVWDY	**15**	16.5 ± 0.8	72.0 ± 1.20
yewacy	**16**	12.8 ± 0.6	53.6 ± 2.50
yewvcy	**17**	14.1 ± 0.7	77.0 ± 4.2
ydwacy	**18**	18.0 ± 0.9	75.1 ± 0.6
ydwvcy	**19**	18.5 ± 0.9	73.4 ± 5.6
control[b]			001.6 ± 0.10
scrambled[c]		10.3 ± 0.1	006.5 ± 0.50

[a] FVIII binding is given as percentage of total bindable material. [b] Uncoated resin. [c] Scrambled sequence ECYYEHWS; 1-Nal is 1-naphthylalanine.

Results and Discussion

Preliminary Studies: Development of Binding Assay and Selection of Lead Structure. For measurement of the FVIII binding ability of the ligands, a microbead assay based on the procedures of Jungbauer and co-workers[59,60] was developed in which the binding of ^{125}I-labeled pdFVIII to the immobilized ligands is measured. The affinity resin Toyopearl AF-Epoxy-650M was chosen as solid support giving best binding results and enabling a chemoselective immobilization of the peptides via a cysteine residue. Kinetic studies of the immobilization reaction showed that the maximal loading density is reached after 2 days of incubation, independent of the peptide sequence and the amounts of peptide used (see Supporting Information). This time frame is also required for the hydrolysis of unoccupied epoxy groups. After various octapeptides from Jungbauer's libraries[59,60] were scanned, the ligand EYHSWEYC (**1**) was selected as the most promising lead candidate, binding ∼50% of the applied FVIII at a loading density of 10.3 μmol/mL of resin (Table 1). The applied FVIII concentration of about 0.7 nM suggested a subnanomolar affinity for FVIII. Besides its high affinity to pdFVIII, the peptide proved even to bind BDD-rFVIII as well as FL-rFVIII with a similar affinity, thus matching our goal to develop a ligand with general applicability (see Table 3). The introduction of an additional spacer molecule (6-aminohexanoic acid, Ahx) did not influence the binding ability [see EYHSWEYAhxC (**2**) in Figure 2], indicating that the peptide does not bind into a deep binding pocket rather than to the surface of factor VIII.

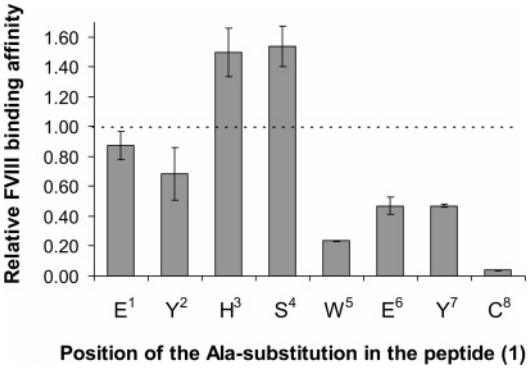


Figure 1. Influence of Ala amino acid substitutions in peptide **1** on the pdFVIII binding affinity.

Therefore, we decided to stay with the microbead binding assay and performed our studies with the immobilized ligands, since the binding of the ligand to FVIII might be significantly affected by the solid support itself or by the presentation of the ligand on the resin surface. As a consequence, the measured effects were the combined results of both the modified sequence itself and influences coming along with changes of, for example, the loading densities and/or an altered presentation of the ligands on the resin surface. This made the systematic combinatorial design more complex but gave us directly the practical binding results of the affinity material, ready to use.

Optimization of the Lead Octapeptide EYHSWEYC (1). To gain basic information about the importance of each amino acid in the sequence for the binding, an Ala and a D-amino acid scan were carried out for peptide **1**. In the Ala scan, each amino acid was subsequently replaced by alanine, thereby deleting the side-chain moiety of the entire amino acid but conserving the chiral information. This experiment (Figure 1) indicated a C-terminal core binding sequence, which was already proposed by Pflegerl et al.[60] Especially the amino acids Trp^5, Glu^6, and Tyr^7 were found to be highly important for efficient pdFVIII binding, as the corresponding substitutions by alanine led to a great decrease of the FVIII binding affinity. In addition, the amino acid Tyr^2 proved to be of importance, while the Ala substitution of Glu^1 did not greatly alter the affinity toward FVIII, and the side-chain residues of His^3 and Ser^4 even seemed to be undesirable for efficient binding. As expected, the peptide with Cys^8 replaced by Ala practically did not immobilize to the resin, resulting in FVIII binding similar to that of uncoated control resin and proving the high selectivity of the immobilization via the cysteine sulfur. In the D-amino acid scan, no significant change in binding affinity was observed for any of the ligands (data not shown). These minor effects of structural modifications were surprising and might be due to the high flexibility of the linear peptide, which enables easy structural adjustment to fit to the FVIII binding site.

Although alanine replacement and D-scanning experiments are useful for identifying important side chains and structural restrictions, they provided little information about the nature of the interactions. Therefore, the effects of single amino acid substitutions with natural and unnatural amino acids providing an isofunctional or isosteric frame, comparable space requirement, or contrasting properties were explored. Binding of FVIII to selected derivatives is presented in Figure 2.

As mentioned above, the substitution of Glu^1 by Ala resulted in a slight decrease of FVIII binding. Similar results were also found for the substitutions by Gln and Asp, whereas a replacement with the sterically demanding Val residue resulted in a great decrease of binding ability. Furthermore, by N-terminal acetylation (ligand **3** in Figure 2) we were able to prove that the N-terminal charge is not important for efficient binding as well, as the corresponding mutant showed equal binding properties. Taken all together, these results were promising in terms of a desired minimization of the peptide sequence.

Tyr^2, in contrast, was found to be quite important for FVIII binding. To determine the nature of its interactions, we replaced Tyr^2 by phenylalanine or O-methylated tyrosine and found a similar loss of FVIII binding ability (~20%) for both mutants. This indicated that Tyr^2 acts as a hydrogen-bond donor via its hydroxyl group.

Following the results of the Ala scan, His^3 and Ser^4 should allow further optimization, since both side-chain residues seemed to be undesirable for efficient FVIII binding. In case of the histidine residue, we found that this is probably due to the basic character of the side-chain residue, as substitution by other basic amino acids like 4-pyridylalanine (4-py) and lysine resulted in a further decrease of the binding affinity. In contrast, replacing His^3 by phenylalanine improves the ability to bind FVIII by almost 40% (see Figure 2). For the Ser^4 residue, the reduced binding affinity of **1** to the corresponding Ala mutant (Figure 1) can be referred to the polar hydroxyl side-chain functionality as the more hydrophobic derivatives, especially the Ala and the Val mutant, led to increased binding properties.

Modifications in the C-terminal part of the molecule proved that this moiety is critical for efficient FVIII binding. All of our mutants at Trp^5 showed at least 50% reduction in FVIII binding ability. Tyr^7, like Tyr^2, could not be replaced by phenylalanine or O-methylated tyrosine without loss of binding affinity, indicating an important hydrogen bridge to this residue. Glu^6 could only be replaced by aspartic acid. The negative charge of the carboxylic group was found to be essential, as shown by the reduced binding affinity of the corresponding glutamine mutant (see Figure 2).

Consequently, we screened numerous combinations of amino acid replacements that gave positive results before, but we did not gain additive effects. Therefore, we stopped our attempts to optimize the sequence and concentrated on a systematic minimization of the peptide.

First Approach in Minimizing EYHSWEYC (1): Development of Hexapeptidic FVIII Ligands. The N-terminal glutamate, as expected, could be deleted without losing binding ability (see Figure 3). In the resulting heptapeptide **4**, however, the substitution of His^3 by phenylalanine (peptide **5**), which resulted in a promising gain of the FVIII binding ability in **1** (see Figure 2), had no effect on the binding ability. An additional truncation of the peptide by removing the important Tyr^2 residue (peptide **6**) accordingly resulted in a noticeable (>30%) loss of binding affinity. To further reduce the sequence, thereby conserving this residue, we placed the C-terminal cysteine linker in the position of the unimportant His^3 residue to obtain the hexapeptide YCSWEY (**7**). This ligand proved to have a high affinity toward FVIII, binding 62% of the applied protein at a loading density of 17.0 μmol/mL.

To optimize **7**, we introduced various substituents (Table 1 and Figure 4): single amino acid replacements showed that Glu^6 can be substituted by Asp (peptide **8**) without affecting the binding affinity, while the substitution of Trp^5 by the more hydrophobic 1-Nal residue (peptide **9**) led to a great loss of the FVIII binding ability, indicating again the critical role of this residue. Substitutions of Ser^4 indicated the preference of a more hydrophobic residue in this position as described above. Thr (peptide **11**) and Ala (peptide **13**) as well as Val (peptide **12**) residues gave remarkable improvements of the binding affinity.

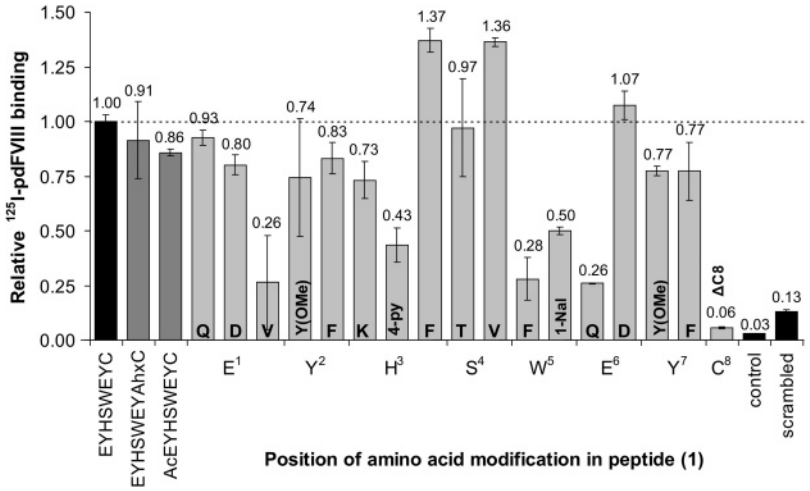


Figure 2. Examples for modifications in the sequence of peptide **1** (reference set as 1.00) and their influence on the FVIII binding affinity (Ahx, 6-aminohexanoic acid; 4-py, 4-pyridylalanine; 1-Nal, 1-naphthylalanine; ΔC8, EYHSWEY; control, uncoated resin; scrambled, ECYYEHWS).

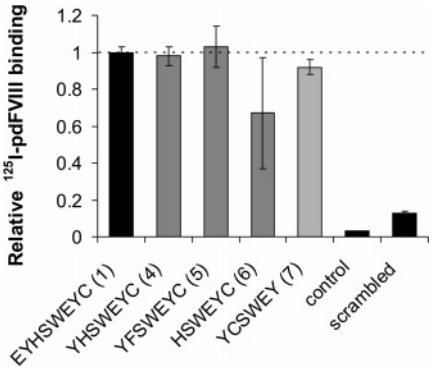


Figure 3. Downsizing of the octapeptidic ligand **1** (reference set as 1.00) to the hexapeptidic ligand **7** (light gray bar). Control, uncoated resin; scrambled sequence, ECYYEHWS.

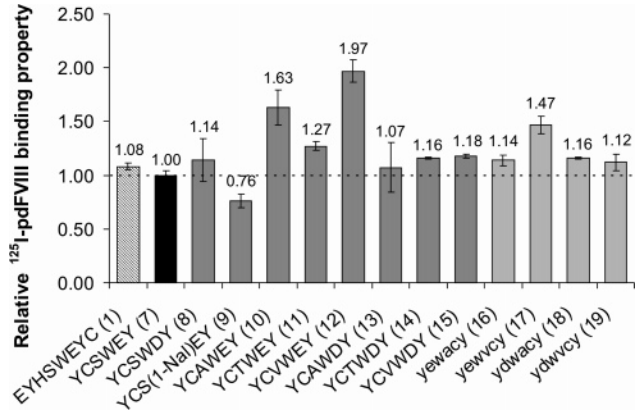


Figure 4. Relative ^{125}I-pdFVIII binding data of mutants of ligand **7** (reference set as 1.00; black bar); dark gray bars represent derivatives with all-L configuration, and light gray bars represent fully retro-inverso peptides (1-Nal, 1-naphthylalanine; lowercase letters denote D-amino acid residues).

The Val-containing peptide **12** showed the best results, binding the applied FVIII almost completely. Surprisingly, the corresponding substitutions of Ser^4 in combination with an Asp residue at position 5 (peptides **13**−**15**) exhibited lower binding affinity.

Having achieved highly potent FVIII ligands, we made an effort to improve the resistance against proteolytic degradation by synthesis of the corresponding fully retro-inverso derivatives, which generally provide enhanced enzymatic stability due to their all-D sequence.[61−65] If the hydrogen bonding and the secondary structure is mirrored, the original side-chain orientation remains conserved but the N- and the C-termini and the direction of the peptide bonds are inverted.[66] We assumed that neither the peptide bonds nor the termini are involved in target interactions. Indeed, the retro-inverso peptides **16**−**19** worked fine and showed only a slightly reduced binding affinity (Table 1 and Figure 4), thus providing us with highly potent factor VIII ligands with improved enzymatic stability as shown below.

However, with regard to our ultimate goal to develop a novel method for FVIII purification that is a real improvement in terms of cost efficiency, this kind of compound was not completely satisfying. The goal was to create a novel kind of ligand that is easily synthesizable on a large scale in solution, thereby avoiding expensive techniques like solid-phase synthesis and HPLC purification to reduce production costs. This is practically not possible for the hexapeptidic ligands. In addition, the unnatural D-amino acid building blocks used for the synthesis of the fully retro-inverso derivatives make this kind of ligand rather expensive.

Second Approach in Minimizing EYHSWEYC (1): Development of Small Molecule FVIII Affinity Ligands. The next goal was the attempt to reduce the initial octapeptide EYHSWEYC (**1**) down to its C-terminal core binding sequence WEYC (**21**, Figure 5). As mentioned above, Glu^1 could be deleted without reducing the FVIII affinity, but the truncation of the Glu^1-Tyr^2 fragment resulted in a 30% loss. However, the larger fragments Glu^1-Tyr^2-His^3 as well as Glu^1-Tyr^2-His^3-Ser^4 could be deleted without additional loss of FVIII binding (peptides **20** and **21**, respectively). Considering the great simplification of the sequence in **21**, the 30% reduced affinity seemed acceptable to proceed with optimizing this new lead structure.

Scanning the minimized lead sequence Trp^{5}Glu^{6}Tyr^{7}Cys,^{8} we found that Tyr^7 cannot be substituted by Phe but Glu^6 can be substituted by Asp, confirming the results described above. Therefore, the work was continued with both alternative residues in position 6, Glu and Asp. In order to optimize the critical Trp^5 residue, we started with an acetylation of its amino function. This modification did not significantly alter the binding affinity of the corresponding tetrapeptides **24** and **25** (see Figure 5), indicating that the amino terminus might not be necessary for efficient binding.

Consequently, Trp^5 was substituted by 3-indolylpropionic acid (3-IPA), which corresponds to a truncation of the amino function

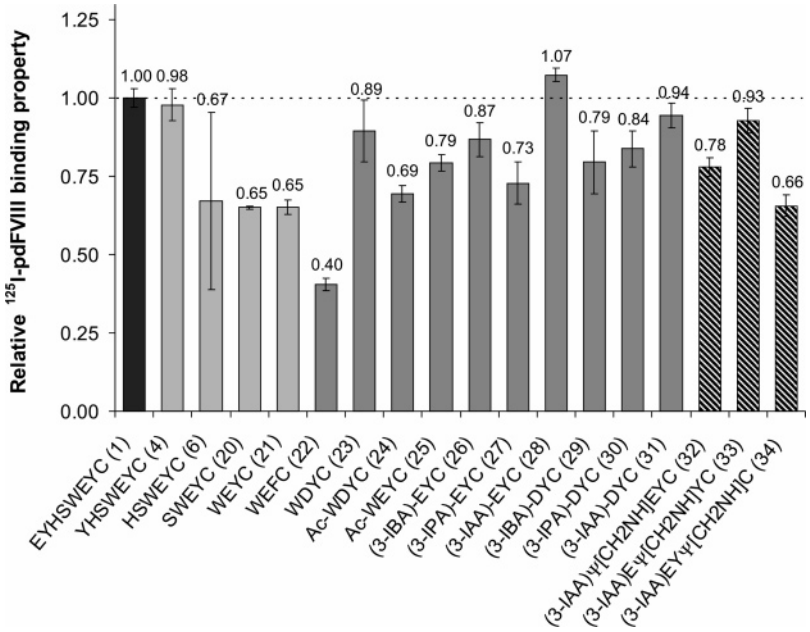
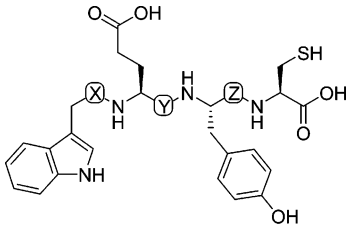


Figure 5. Relative binding data of minimized mutants of ligand **1**. Stepwise minimization of **1** (black bar) to its core binding motive WEYC (**21**) (light gray bars), side-chain optimization of **21** (dark gray bars), and backbone modification of **28** (hatched bars) are shown.

Table 2. Absolute Binding Data of Selected Small Molecule FVIII Ligands

sequence	no.	ligand loading (μmol/mL)	^{125}I-pdFVIII binding[a] (%)
WEYC	**21**	22.7 ± 0.5	46.4 ± 1.7
WDYC	**23**	20.5 ± 1.0	62.8 ± 6.9
(3-IAA)EYC	**28**	23.4 ± 1.2	76.7 ± 1.6
(3-IAA)DYC	**31**	24.3 ± 0.7	67.7 ± 2.8
(3-IAA)ψ[CH_2NH]EYC	**32**	18.4 ± 0.9	53.7 ± 1.9
(3-IAA)Eψ[CH_2NH]YC	**33**	20.8 ± 1.0	65.3 ± 3.0
(3-IAA)EYψ[CH_2NH]C	**34**	16.0 ± 0.8	43.4 ± 2.3

[a] FVIII binding is given as percentage of total bindable material.



32 X = CH_2, Y = CO, Z = CO
33 X = CO, Y = CH_2, Z = CO
34 X = CO, Y = CO, Z = CH_2

Figure 6. Structures of the small molecule factor VIII ligands **32**−**34** with modified peptide bonds.

of tryptophan. This modification led to a slight increase of the affinity toward FVIII in both sequences, (3-IPA)EYC (**27**) and (3-IPA)DYC (**30**). Subsequently, the length of the side chain was varied to optimize the position of the indole residue by substituting 3-IPA with 3-indolylacetic acid (3-IAA) and 3-indolylbutyric acid (3-IBA). This experiment indicated a tendency of better binding properties with shorter side chain for the Asp-containing derivative, but a significant improvement could not be achieved (compare ligands **29**−**31** in Figure 5). In contrast, for the corresponding derivatives with a glutamic acid in position 6 (ligands **26**−**28**), the substitution of 3-indolylpropionic acid by 3-indolylacetic acid (ligand **28**) led to a 50% increase of the affinity toward FVIII, binding 77% of the applied ^{125}I-pdFVIII at a ligand loading of 23 μmol/mL (Table 2).

We now focused on optimization of resistance against proteases and substituted subsequently all peptide bonds in **28** by a reduced peptide bond (CH_2−NH), producing ligands **32**−**34** (Figure 6). These unnatural peptides were synthesized on solid phase[67,68] by a 9-fluorenylmethyloxycarbonyl (Fmoc) strategy[69−71] on trityl chloride polystyrol (TCP) resin[72] with 1-hydroxybenzotriazole (HOBt) and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU)[73,74] as coupling reagents. The CH_2−NH bonds were introduced via reductive alkylation following a procedure of Krchnak et al.[75] Exploring the binding properties of the unnatural ligands, we found that the substitutions of the N-terminal peptide bond connecting 3-IAA and Glu[6] (compound **32**) or of the C-terminal peptide bond connecting Tyr[7] and Cys[8] (compound **34**) both led to a significant loss of affinity, indicating that these peptide bonds are involved in FVIII interactions. In contrast, the modification of the central peptide bond (*Y* in Figure 6) connecting Glu[6] and Tyr[7] (compound **33**) had little effect on the FVIII binding (see Figure 5 and Table 2). As the Glu−Tyr bond might be cleaved by matrix metalloproteinase 3 (MMP-3), this substitution might significantly contribute to enhanced resistance against enzymatic degradation.[76] Due to its high affinity and the expected very high enzymatic stability, the peptidomimetic ligand **33** was chosen as the most promising ligand for further evaluation.

Verification of the Enzymatic Stability. The resistance against enzymatic degradation was determined by treating the ligands with fresh human serum (single donor) and the amount of intact ligand was determined by quantitative HPLC and HPLC-MS analysis. For our study the octapeptide **1** as well as the fully retro-inverso peptide **19** and the peptidomimetic **33** were chosen. As shown in Figure 7A, the octapeptide **1** is completely degraded after 3 h of incubation with serum, showing its limited applicability as affinity ligand. As assumed, the fully retro-inverso peptide **19** showed a significantly enhanced resistance against enzymatic degradation. Nevertheless, despite its unnatural structure it was slowly decomposed to a final content of 40% unchanged ligand after 3 h of treatment. In contrast, the small peptidomimetic **33** proved to be highly stable in human serum. We did not observe any conversion within

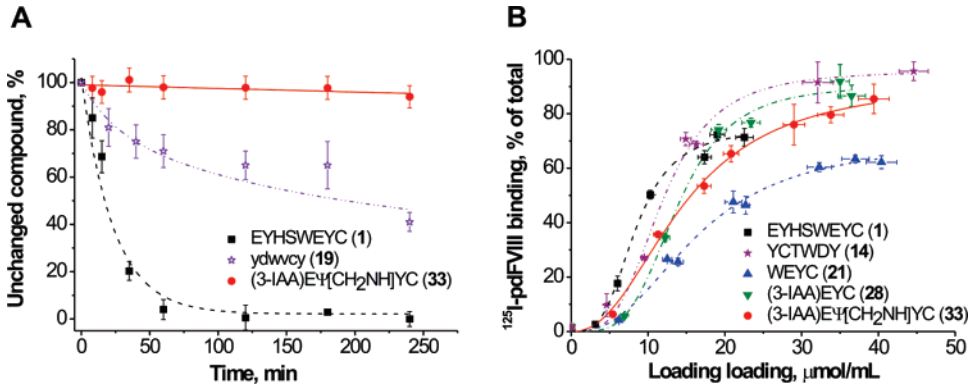


Figure 7. Evaluation of ligand stability and FVIII binding properties. (A) Determination of the stability of octapeptide EYHSWEYC (**1**), fully retro-inverso peptide **19**, and the minimized peptidomimetic **33** against enzymatic degradation by treatment with fresh human serum (single donor). (B) Effect of the ligand loading on the capture of ^{125}I-pdFVIII.

3 h of incubation in human serum (Figure 7A), which is a major improvement over previous FVIII ligands.

Effects of Ligand Density on FVIII Binding. The ability to capture proteins with an affinity resin greatly depends on ligand properties as well as on its concentration on the resin.[77] To determine the effects of the latter, we prepared resins of different loading density of the two best small ligands, **28** and **33**, and the high-binding hexapeptide **14** to measure their ability to capture FVIII (Figure 7B). The initial octapeptide EYHSWEYC (**1**) as well as the tetrapeptide WEYC (**21**) served as references.

As expected, an increase of ligand density resulted in an increase of FVIII adsorption. However, there is a minimum concentration of about 5−10 μmol of ligand/mL of resin (depending on the nature of the ligand) that is crucial for efficient binding, and saturation was observed at high loading densities. The data points for each compound could be fitted by a Hill function, confirming this observation. Another limit was found for the maximal reachable ligand density. Here, a significant difference between the lead octapeptide **1** and the minimized ligands **14**, **21**, **28**, and **33** was observed. While a loading density of 25 μmol/mL could not be exceeded for **1**, even if a vast excess of ligand was applied, the minimized compounds readily immobilized up to 40 μmol/mL. At these high loading densities, the small peptidomimetics **28** and **33** bound almost 90% and the hexapeptide **14** bound 95% of the applied FVIII, in contrast to **1**, where a maximum of 70% binding could not be exceeded.

Binding of Recombinant FVIII Molecules. The different factor VIII molecules (pdFVIII, FL-rFVIII, and BDD-rFVIII) vary in their structure and glycosylation.[78,79] Thus, a binding site independent from glycosylation and outside the B domain was a basic requirement to fulfill our goal of a broad applicability of our ligands to the purification of all biotechnologically relevant FVIII products. As mentioned above, the lead compound **1** was found to bind to such site, as it binds to various FVIII molecules with similar affinity. Minimized derivatives were also checked for their binding to these FVIII molecules, and their high affinity to FL-rFVIII as well as to BDD-rFVIII was confirmed. A selection of results is presented in Table 3.

Solution-Phase Synthesis of 33. Altogether, the peptidomimetic **33** fulfilled all requirements for a FVIII affinity ligand and was found to be the most promising candidate for the purpose of purifying FVIII. Therefore we decided to develop a solution-phase synthesis for **33** to allow cost-efficient production on a preparative scale for further experiments.

The synthesis of peptides from the C-terminus to the N-terminus is the most common strategy.[80] However, the inverse direction seemed more favorable in this special case: In peptide synthesis the cysteine side chain is most commonly protected with a trityl (Trt) group, as this group is easily removable under acidic conditions.[81] In the case of a C to N synthesis, Cys would be introduced in the first step. This is not recommended for a multistep synthesis that includes several washing and chromatography columns, since the Cys side-chain protection group is acid-labile. In addition, the cysteine amino acid itself is very sensitive toward racemization.[82] The main disadvantage of the N to C direction is the risk of racemization by oxazolone formation.[83,84] However, this requires a C-activated N-acylamino acid, an arrangement that is not present in our N to C procedure for the synthesis of **33**, as described below.

Table 3. Binding Data for Recombinant Factor VIII Molecules

sequence	no.	ligand loading (μmol/mL)	^{125}I-FL-rFVIII binding[a] (%)	^{125}I-BDD-rFVIII binding[a] (%)
EYHSWEYC	**1**	8.8 ± 0.4	40.8 ± 0.8	35.0 ± 7.2
YCTWEY	**11**	16.3 ± 0.8	57.8 ± 3.7	62.9 ± 8.6
(3-IAA)EYC	**28**	19.2 ± 1.0	54.9 ± 6.3	49.6 ± 1.7
(3-IAA)Eψ[CH$_2$NH]YC	**33**	17.3 ± 0.9	53.3 ± 2.3	40.6 ± 2.3

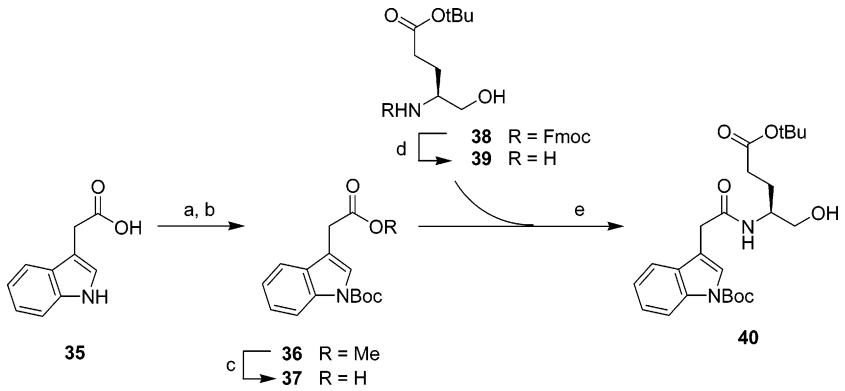
[a] FVIII binding is given as percentage of total bindable material.

Accordingly, we started the synthesis from 3-indolylacetic acid (**35**). The indole nitrogen of **35** was protected with a *tert*-butyloxycarbonyl (Boc) group in a three-step reaction sequence according to literature procedures (Scheme 1): **35** was transformed into its methyl ester by treatment with $SOCl_2$ in MeOH and the indole nitrogen was subsequently protected with a Boc group by reaction with *tert*-butyl dicarbonate and 4-dimethylaminopyridine (DMAP)[85] in acetonitrile to achieve **36**.[86−88] Saponification then produced the desired indolylacetic acid **37** with 70% overall yield.

The conversion to **40** was achieved by coupling **37** to the side-chain-protected glutamol **39** with HOBt/TBTU as coupling reagents. Compound **39** was readily available from commercial Fmoc-glutamol(OtBu) (**38**) by treatment with piperidine and could be used without further purification. For the coupling to **37**, protection of the free hydroxyl functionality in **39** was not necessary and the reaction proceeded cleanly to give the N-substituted glutamol **40** in 95% yield.

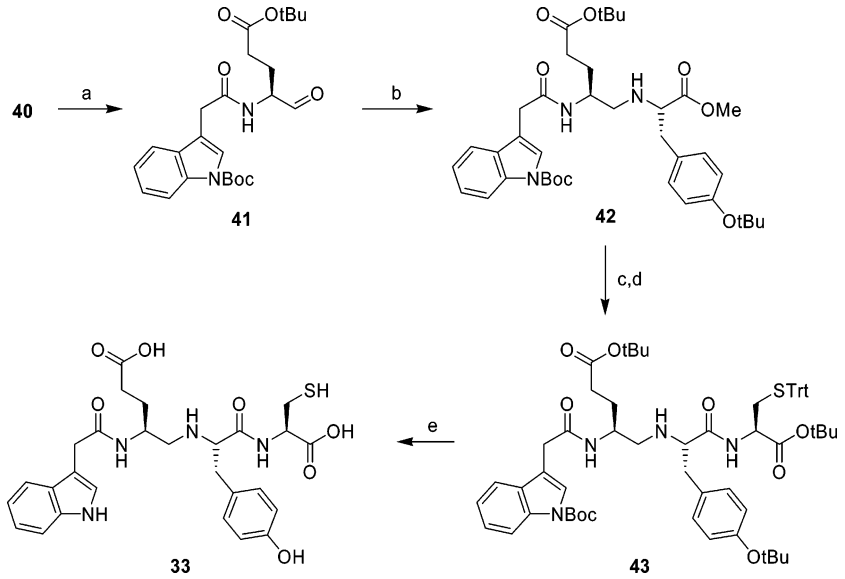
The reduced peptide bond linking the glutamic acid and tyrosine residues in the target compound **33** was formed by a reductive alkylation of the corresponding aldehyde **41** and commercial Tyr(tBu)OMe (Scheme 2). Unfortunately, the first experiments for the synthesis of the secondary amine **42** led to an inseparable 8:2 mixture of diastereomeric isomers due to racemization of the highly sensitive aminoaldehyde **41**. We found that this was due to the basic reaction conditions during

Scheme 1. Synthesis of the N-Substituted Glutamol **40**[a]



[a] Reagents and conditions: (a) $SOCl_2$, MeOH, 18 h, 98%. (b) Boc_2O, DMAP, acetonitrile, 4 h, 86% (two steps). (c) LiOH, THF, MeOH, H_2O, 18 h, 83%. (d) piperidine, DMF, 1 h. (e) HOBt, TBTU, DIPEA, 0 °C → rt, 4 h, 95% (two steps).

Scheme 2. Synthesis of the Tetrapeptide Mimetic **33**[a]



[a] Reagents and conditions: (a) Dess-Martin periodinane, DCM, 6 h. (b) (1) Tyr(tBu)OMe·HCl, $MgSO_4$, DCM, 30 min; (2) $NaB(OAc)_3H$, 18 h, 75% (two steps). (c) LiOH, THF, dioxane, H_2O, 1.5 h. (d) Cys(Trt)OtBu·HCl, HOBt, TBTU, 2,4,6-collidine, 10 °C → rt, 18 h, 83% (two steps). (e) TIPS, H_2O, TFA 0 → 95%, 8 h, 98%.

the aldehyde formation by Swern oxidation[89,90] with the strong base *N*,*N*-diisopropylethylamine (DIPEA).[91,92] Also, the further transformation to the imine by treatment with Tyr(OtBu)OMe·HCl was done in the presence of DIPEA and $MgSO_4$.[93] Racemization was completely overcome by the use of Dess−Martin periodinane oxidation[94−96] instead of Swern oxidation and short preformation of the imine in situ in the absence of base, rapidly followed by addition of the reducing agent. This procedure gave the desired secondary amine **42** in 75% yield over two steps. The methyl ester in **42** was cleaved by saponification and the resulting free acid was coupled to Cys(Trt)OtBu·HCl[97] in the presence of HOBt/TBTU and the mild base 2,4,6-collidine to yield **43** (83% yield, two steps). Although the cysteine *tert*-butyl ester we used is not commercially available, it was favored over the commercial methyl ester as it is readily synthesizable[97] and it allows a one-step deprotection of **43** to the desired free peptidomimetic **33** under acidic conditions.

The final deprotection and purification is the critical step in terms of an economic production of **33**. Therefore, we concentrated on optimization of the deprotection reaction in order to reduce byproduct formation to avoid a purification of the final product. The key to minimize side product formation was to avoid a sudden high concentration of *tert*-butyl cations, as it occurs if **43** is directly treated with the cleavage mixture [95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS), and 2.5% H_2O].[98] Thus, the problem was solved by suspending **43** in a vigorously stirred mixture of the scavengers water and TIPS (1:1) and slowly adding the TFA over a period of 8 h to a final concentration of 95%. By this procedure the byproduct formation (typically 15% as determined by HPLC) was significantly reduced to obtain the final peptidomimetic **33** in 98% yield (purity > 95%) after precipitation in ether/pentane. To prove the diastereomeric purity, we synthesized all possible diastereomers of **33** and analyzed them by HPLC. Coinjection with **33** gave baseline separation for all diastereomers (data not shown).

Purification of pdFVIII by Use of Peptidomimetic 33-Coated Resin. To demonstrate the potential of peptidomimetic **33** as affinity ligand for FVIII purification, we performed a sample purification from medium. FVIII adsorption and elution was accomplished by two buffers varying only in their NaCl content (see Supporting Information). This is an indication that the interaction between FVIII and the evaluation peptides has predominantly ionic character, as observed for various antibodies before.[99,100] The eluted protein fractions were analyzed by determining OD_{280} as well as by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on 10% gels

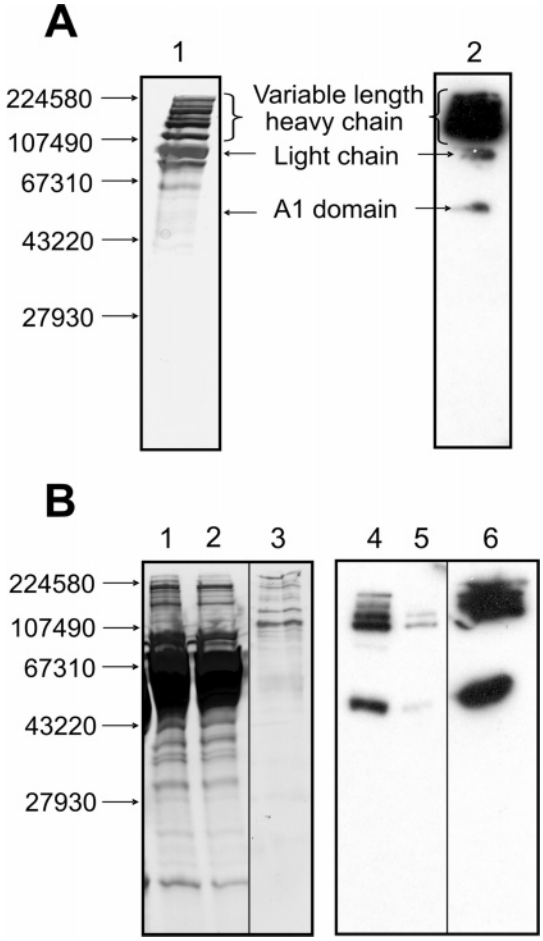


Figure 8. Purification of pdFVIII using peptidomimetic **33**-coated resin. Samples from fractions were analyzed by SDS−10% PAGE followed by silver staining or Western blotting with mAb C5 and mAb 413 against FVIII. (A) SDS−PAGE (lane 1) and Western blot (lane 2) of pure pdFVIII. (B) SDS−PAGE and Western blot from the purification from cell-conditioned FBS-containing medium spiked with 0.5 mg of pdFVIII on a 1 mL column packed with peptidomimetic **33**-coated resin. SDS−PAGE: lane 1, source solution (medium with pdFVIII); lane 2, flowthrough; lane 3, elution fraction. Western blot: lane 4, source solution (medium with pdFVIII); lane 5, flowthrough; lane 6, elution fraction.

followed by silver staining or Western blotting with monoclonal antibodies against FVIII in order to determine the origin of the bands. Figure 8A shows SDS−PAGE (lane 1) and Western blot (lane 2) analysis of a pure pdFVIII sample that was used as reference. The following bands were observed: heterogeneous 230−90 kDa heavy-chain bands and the ∼80 kDa light-chain doublet bands. In addition, we found traces of proteolytic bands: ∼73 kDa activated light-chain doublet bands, a ∼50 kDa band of A1 domain that is highly reactive against mAb C5, and a ∼43 kDa band (A2 domain).

The high efficiency of ligand **33**-coated resin in FVIII affinity purification was proven by a purification of pdFVIII from cell-conditioned fetal bovine serum- (FBS-) containing Dulbecco's modification of Eagle's medium (DMEM) that was spiked with FVIII as shown in Figure 8B. Lanes 1 and 4 show the SDS−PAGE and Western blotting of the source solution, which was loaded on a column packed with **33**-coated resin. The corresponding analysis of the flowthrough is given in lanes 2 and 5, which clearly demonstrate that the majority of contaminant proteins, presented in vast excess to FVIII, were eluted in the flowthrough (compare lanes 1 and 2), which contained only traces of FVIII (compare lanes 4 and 5). After additional washings, FVIII was eluted from the column (see lanes 3 and 6) with a mild buffer to afford highly satisfactory FVIII retention (89%) and 63-fold concentration.

Conclusion

Two groups of affinity ligands, hexapeptidic and small peptidomimetic ligands, each showing specific advantages, were described. Their choice for application in FVIII affinity purification processes will depend on process characteristics and/or the objective. The best hexapeptides proved to have a superior FVIII binding profile than the small peptidomimetics, while suffering from lower enzymatic stability and higher production costs comparing with the small peptidomimetics. However, in specific applications, in particular if extremely high FVIII binding properties are needed, these peptide ligands will be the material of choice.

The small ligands, in particular peptidomimetic **33**, proved to have the best overall performance. Ligand **33** readily immobilized to the Epoxy Toyopearl resin to achieve resins of high ligand loading strongly binding FVIII. We also demonstrated its very high resistance against enzymatic degradation. A solution synthesis was developed for **33**, allowing an easy and cost-efficient production in preparative scale and with reasonable cost, in contrast to the complex preparation of mAbs by recombinant techniques. As the synthesis is performed without use of any materials of human or animal origin, this ligand does also not carry a risk of transmitting pathogens. In addition, the synthetic origin of ligand **33** offers opportunities for easy modification of the ligand's structure. This can be used, for example, to further optimize the ligand in terms of affinity, stability, loading capability, or elution conditions, which is not trivial for antibodies. The applicability of the ligands was demonstrated by affinity purification of FVIII from FBS-containing medium by use of **33**-coated resin, achieving FVIII in high yield and purity.

Hence, by use of our novel ligands to replace antibody in affinity purification, the FVIII manufacturing costs might be significantly reduced and safety concerns regarding to the application of antibodies are overcome. Moreover, the ligands were proven to bind all FVIII variants, thus being suitable for current and next-generation FVIII products.

Experimental Section

(*S*)-*tert*-Butyl 4-[2-(1-*tert*-Butyloxycarbonylindol-3-yl)]-5-hydroxypentanoate (40). Fmoc-Glutamol(OtBu) (**38**) (1.83 g, 4.45 mmol) was dissolved in piperidine/*N*,*N*-dimethylformamide (DMF) (20/80 v/v; 100 mL), and after being stirred for 1 h, the mixture was evaporated to dryness. The resulting crude amine **39** was taken up in DMF (250 mL), and **36** (1.23 g, 4.45 mmol, 1.0 equiv), HOBt (681 mg, 4.45 mmol, 1.0 equiv), and TBTU (1.43 g, 4.45 mmol, 1.0 equiv) were added. Then the mixture was cooled to 0 °C and DIPEA (2.25 mL, 13.2 mmol, 3.0 equiv) was added. After the mixture was stirred for 18 h at room temperature, the solvent was removed in vacuo and the residue was taken up in EtOAc (300 mL). The mixture was washed with saturated aqueous NH_4Cl (2 × 150 mL), saturated aqueous $NaHCO_3$ (2 × 150 mL), and brine (150 mL). After the mixture was dried over Na_2SO_4, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel ($MeOH/CHCl_3$; gradient 1:50 → 1:20) to give **40** (1.88 g, 95%) as a hygroscopic colorless oil.

(*S*)-*tert*-Butyl 4-[2-(1-*tert*-Butyloxycarbonylindol-3-yl)acetylamino]-4-formylbutanoate (41). To a solution of **40** (1.2 g, 2.68 mmol, 1.0 equiv) in dry DCM (30 mL) was added Dess−Martin periodinane (3.41 g, 8.04 mmol, 3.0 equiv) in three portions over 1.5 h. The mixture was allowed to stir for another 1 h, after which

the suspension was diluted with DCM (30 mL) and subsequently washed with a mixture of 10% aqueous $Na_2S_2O_3$ and saturated aqueous $NaHCO_3$ (1:1 v/v; 2 × 30 mL), water (30 mL), and brine (30 mL). Drying over Na_2SO_4 and evaporation gave the crude aldehyde **41** (1.18 g) as a pale yellow solid, which was directly used without further purification.

(*S*)-*tert*-Butyl 5-[(*S*)-1-Methoxycarbonyl-2-(4-*tert*-butoxyphenyl)ethylamino]-4-[2-(1-*tert*-butyloxycarbonyl-indol-3-yl)acetylamino]pentanoate (42). The crude aldehyde **41** (1.18 g) was dissolved in dry DCM (60 mL), and Tyr(OtBu)OMe·HCl (925 mg, 3.21 mmol, 1.2 equiv) and $MgSO_4$ (1.61 g, 13.4 mmol, 5.0 equiv) were added. After 30 min of reaction, $NaB(OAc)_3H$ (3.18 g, 15.0 mmol, 5.6 equiv) was added and stirring was continued for an additional 18 h. Then the mixture was diluted with saturated aqueous $NaHCO_3$ (50 mL) and, after 30 min, extracted with DCM (2 × 60 mL). The combined organic layers were dried over Na_2SO_4, the solvent was removed under reduced pressure, and the crude product was purified by flash chromatography on silica gel (EtOAc/hexane gradient 1:2 → 2:1) to give **42** (1.37 g, 75% based on **40**) as a pale yellow oil.

(*S*)-*tert*-Butyl 5-{(*S*)-1-[(*S*)-1-Butoxycarbonyl-2-tritylsulfanylethylcarbamoyl]-2-(4-butoxyphenyl)ethylamino}-4-[2-(1-*tert*-butyloxycarbonylindol-3-yl)acetylamino]pentanoate (43). A solution of LiOH·H_2O (196 mg, 4.68 μmol, 3.5 equiv) in H_2O (5.3 mL) was added to a solution of **42** (1.06 g, 1.56 mmol, 1.0 equiv) in tetrahydrofuran (THF) (5.3 mL) and dioxane (15.9 mL). After being stirred for 1.5 h, the mixture was acidified by addition of a 10% aqueous solution of citric acid (100 mL) and the aqueous layer was extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with water (100 mL) and brine (50 mL), dried (Na_2SO_4), and evaporated to give the crude acid [986 mg; 93% purity; RP-HPLC (20% → 100%) R_t = 23.0] as a pale yellow solid, which was redissolved in DMF (55 mL). Then Cys(Trt)O*t*Bu·HCl[97] (1.06 g, 2.34 mmol, 1.5 equiv), HOBt (232 mg, 1.72 mmol, 1.1 equiv), and TBTU (552 mg, 1.72 mmol, 1.1 equiv) were added and the mixture was cooled to 10 °C. 2,4,6-Collidine (1.03 mL, 7.80 mmol, 5.0 equiv) was added and the solution was stirred for 18 h at room temperature. After the solvent was evaporated, the residue was taken up in EtOAc (200 mL) and subsequently washed with saturated aqueous NH_4Cl (2 × 150 mL), saturated aqueous $NaHCO_3$ (2 × 150 mL), and brine (150 mL). After the mixture was dried over Na_2SO_4, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (EtOAc/hexane; gradient 1:4 → 2:1) to give **43** (1.38 g, 83%) as a hygroscopic, pale yellow oil.

5-[1-(1-Carboxy-2-mercaptoethylcarbamoyl)-2-(4-hydroxyphenyl)ethylamino]-4-(2-1*H*-indol-3-ylacetylamino)pentanoic acid (33). To a vigorously stirred mixture of **43** (500 mg, 0.47 mmol) in triisopropylethylsilane (250 μL; 2.5%) and water (250 μL; 2.5%) was added trifluoroacetic acid (9.50 mL; 95%) slowly over 8 h. The mixture was stirred for an additional 30 min, after which the solvent was removed in vacuo. The residue was dissolved in a minimum volume of acetic acid and precipitated in ice-cold ether/pentane (2 × 20 mL) to give pure **33** (242 mg, 93%).

Immobilization of Ligands on Toyopearl AF-Epoxy-650M Resin. Ligands (2.5 mg) were dissolved in 0.25 mL of immobilization buffer (0.2 M sodium bicarbonate, pH 10.3), and Toyopearl AF-Epoxy-650M resin (36 mg, corresponding to 0.125 mL of swollen resin; Tosoh Bioscience, Stuttgart, Germany) was added, followed by gentle rotation for 48 h at room temperature. Then the resin was washed with immobilization buffer (once), 1 M NaCl (once), and binding buffer (three times; 0.01 M Hepes, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.01% Tween-80, pH 7.4). The achieved loading density was determined by measuring the UV adsorption (280 nm) of the source solution before and after immobilization of the ligands.

Microbead Binding Assay. The ligand-coated resin was resuspended in binding buffer (1:7 v/v) and aliquotted into Eppendorf tubes (40 μL/tube). ^{125}I-pdFVIII, ^{125}I-FL-rFVIII, or ^{125}I-BDD-FVIII (100 000 cpm in 10 μL; ~25 ng of FVIII) was added to the tubes, and the volume of the mixture was adjusted to 100 μL with binding buffer containing 4% bovine serum albumin (BSA). After 2 h of incubation at room temperature on a rotator, the samples were washed with binding buffer (four times) and the radioactivity of the beads was measured. The radioactivity of uncoated resin was considered as background value, and binding was calculated as a percent of maximal achievable binding, as determined by immunoprecipitation of labeled FVIII with anti-FVIII mAb 8860-coated resin. Scrambled peptide (ECYYEHWS) was used as negative control and peptide **1** (EYHSWEYC) as positive control in each experiment. Presented data are the mean values of duplicate experiments of two independently prepared samples (four determinations overall).

Affinity Purification of pdFVIII on Peptidomimetic 33-Coated Resin: 1. General. Peptidomimetic **33**-coated resin (1 mL) was prepared from 25 mg (37 μmol) of ligand **33** and 360 mg of dry Toyopearl AF-Epoxy-650M resin as described above. The resulting wet resin (ligand loading 21 μmol/mL) was packed into a glass column (GE Healthcare, Piscataway, NJ) and purification was performed by employing a Waters 650E Advanced Protein Purification System (Waters, Milford, MA) at a flow rate of 1 mL/min with the following buffers: buffer 1 was 0.01 M Hepes, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.01% Tween-80, and buffer-2 was 0.01 M Hepes, 1 M NaCl, 5 mM $CaCl_2$, and 0.01% Tween-80. The elution was monitored by a flowthrough UV detector (Waters 490 E) at 280 nm (OD_{280}).

2. Affinity Chromatography. pdFVIII (0.5 mg), previously purified as described above, was mixed with cell-conditioned 10% FBS-containing DMEM, which was diluted with 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 5 mM $CaCl_2$, and 0.01% Tween-80 to a final salt concentration of 0.1 M NaCl. The mixture was applied onto the column, followed by wash with pure buffer 1 until the OD_{280} returned to background and then a mixture of buffers 1 and 2 (85:15 v/v). Then, FVIII was eluted with a mixture of 40% buffer 1 and 60% buffer 2. The control experiment without added medium was performed by the same method except that FVIII was eluted directly after wash with buffer 1 by a mixture of 20% buffer 1 and 80% buffer 2.

3. Analysis of Elution Fractions. Elution fractions were analyzed by determining OD_{280} as well as by SDS−PAGE on 10% gels followed by silver staining or Western blotting as described in the Supporting Information.

Acknowledgment. We thank Martin Haslbeck, Gerd Gemmecker, and Jayanta Chatterjee for helpful discussions and Mona Wolff, Burghard Cordes, and Helmut Krause for their technical assistance. Financial support by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie is gratefully acknowledged.

Supporting Information Available: Synthesis and characterization of **36** and **37**, analytical data of **40**−**43** and **33**, general procedures for solid-phase synthesis of peptidic and peptidomimetic ligands, analysis of immobilization kinetics, ^{1}H and ^{13}C NMR spectra of new compounds, and description of ^{125}I-labeling of FVIII, SDS−PAGE and Western blotting. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM070304X