A Useful Disulfide Linker for Single-Bead Analysis of Peptide Libraries

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A disulfide-linker for conventional peptide synthesis, attached to a PEGA-resin, has been developed. Reductive hydrolysis cleaves the linker within minutes, liberating the synthesized peptide for rapid sequencing by tandem mass spectrometry. The method has been tested for ten peptides in a single-bead fashion.

1. Introduction. – The efficiency of combinatorial chemistry relies to a great extent on suitable linkers. Linkers carrying photosensitive 2-nitrobenzyl groups [1–4] or a pivaloyl glycol moiety [5], as well as acid-sensitive linkers in the *Wang* resin [6], *SASRIN* [7], or the *Rink* resin [8] are well-established and commonly used. The disadvantages of photolinkers are often too-long irradiation times at wavelengths that may be critical to the stability of the product as well as the formation of the reactive 2-nitrosobenzaldehyde. On the other hand, problems arise with acid-sensitive linkers due to side-reactions caused by trifluoroacetic acid (TFA) upon cleavage, namely alkylation of methionine or tyrosine residues, modification of tryptophan residues by the linker, by side-chain protecting groups, or by scavengers, as well as partial destruction of other sensitive groups found in peptides. Scavengers tend to suppress these effects, yet their presence leads to contamination of the final product. Furthermore, acidic cleavage often suffers from long exposure times, laborious protocols, or incomplete hydrolysis due to partial neutralization by amide bonds [9].

The aim of our investigation was to develop a new linker that could be cleaved within minutes from a biocompatible PEGA resin[1]) for screening large single-bead peptide libraries in short time. The new linker was chosen on the basis of earlier results. *Bannwarth* and *Wippler* [10] have employed a disulfide-containing 'purification handle' for oligonucleotide synthesis on controlled-pore glass as solid support; and *Brugidou* and *Méry* [11] have used 2-[2-hydroxypropyl)disulfanyl]isobutyric acid as a linker for solid-phase peptide synthesis. However, the latter approach suffered from a rather elaborate reaction protocol, from too-long cleavage times, and it has not been applied to single-bead analysis.

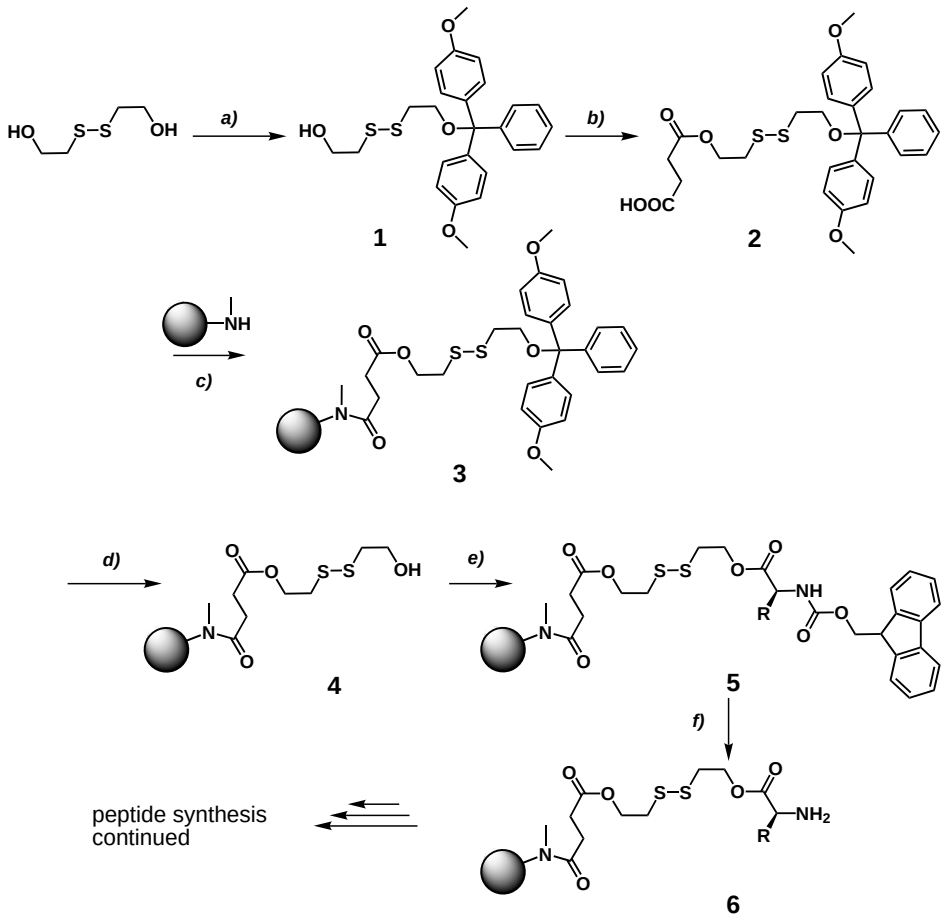
2. Results and Discussion. – The linker to be attached to the PEGA bead was prepared from mono-protected 2-[(2-hydroxyethyl)disulfonyl]ethanol (**1**), which was elongated with succinic anhydride to yield **2**. The latter was coupled to the free NH_2

[1]) PEGA stands for polyamide/poly(ethylene glycole) amine.

group of the resin, leading to **3**. Removal of the dimethoxy-substituted trityl protecting group (DMT) liberated the alcohol **4**, to which the first Fmoc-protected[2]) amino acid was coupled to furnish **5** (*Scheme 1*). *N*-Deprotection of **5** gave the PEGA bead **6**, ready for peptide synthesis under standard conditions. Thereby, the removal of the DMT group (**3** → **4**) helped to determine the epitopic density of the resin. In general, we obtained loadings of 0.1 – 0.17 mmol/g resin.

To find the optimum cleavage conditions for the S_2-linker, the resin-bound dipeptide **7**, carrying a dabsyl[3]) chromophore ($\varepsilon_{466} = 33000$), was used. As shown in

Scheme 1



a) DMTCl, pyridine, 2 h, r.t., 55%. *b*) Succinic anhydride, DMAP, pyridine, CH_2Cl_2, 24 h. r.t., 66%. *c*) HATU, HOAt, DIPEA, NMP, 16 h, r.t. *d*) TFA, H_2O, $(i\text{-}Pr)_3SiH$, 30 min, r.t. *e*) Fmoc glycine, DCC, DMAP, pyridine, 3 h, r.t. *f*) HATU, HOAt, DIPEA, NMP, 90 min, r.t. For abbreviations, see *Exper. Part*.

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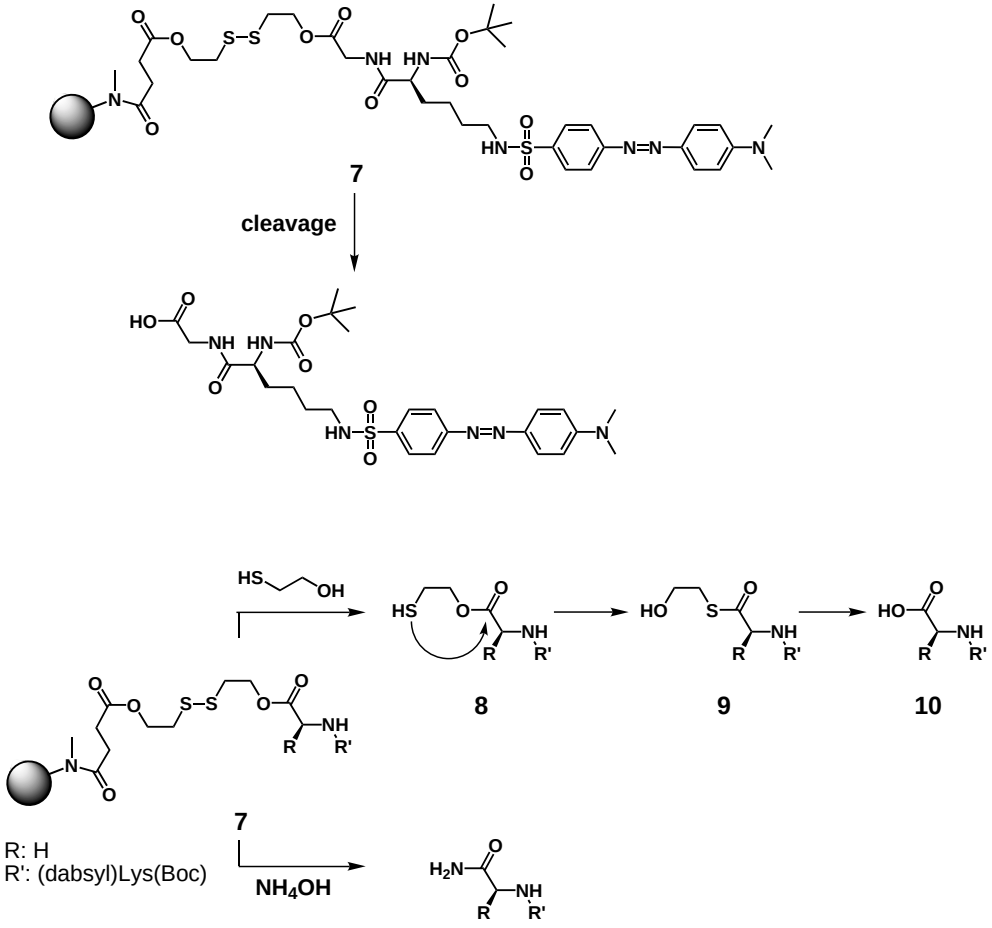
[2]) Fmoc represents the [(9*H*-fluoren-9-yl)methoxy]carbonyl group.

[3]) Dabsyl stands for the 4-{[-(dimethylamino)phenyl]diazenyl}benzenesulfonyl group.

Scheme 2, the linker can be cleaved either with NH_4OH (15% in H_2O (pH 12)/MeCN) to yield the terminal amide of the synthetic peptide, or by using a 0.1M 2-mercaptoethanol solution containing 0.2% of NH_4OH (H_2O (pH 10)/MeCN 1:1). Under the latter conditions, the S_2-group of **7** is reduced to **8**. The liberated SH moiety then attacks the *C*-terminus of the peptide, and the intermediate thioester **9** is hydrolyzed to **10**. This mechanism was supported by HPLC-analysis during the cleavage of the dabsyl-labelled peptide **7**. Both the final product **10** as well as the intermediates **8** and **9** were observed, separated, and identified by their characteristic IR spectra.

Scheme 2. *Cleavage of the Disulfide-Linker of a Dabsyl-Chromophore-Containing Peptide Under Reductive (100 mM Mercaptoethanol in MeCN) and Basic Conditions* (25% of aq. NH_4OH soln. in MeCN)



Conceptually, reductive cleavage under basic conditions is particularly attractive because *i*) the reaction takes only *ca.* 2 min (see *Fig. 1*), and *ii*) the linker eliminates itself while the synthesized peptide is set free and can be directly analyzed by MS.

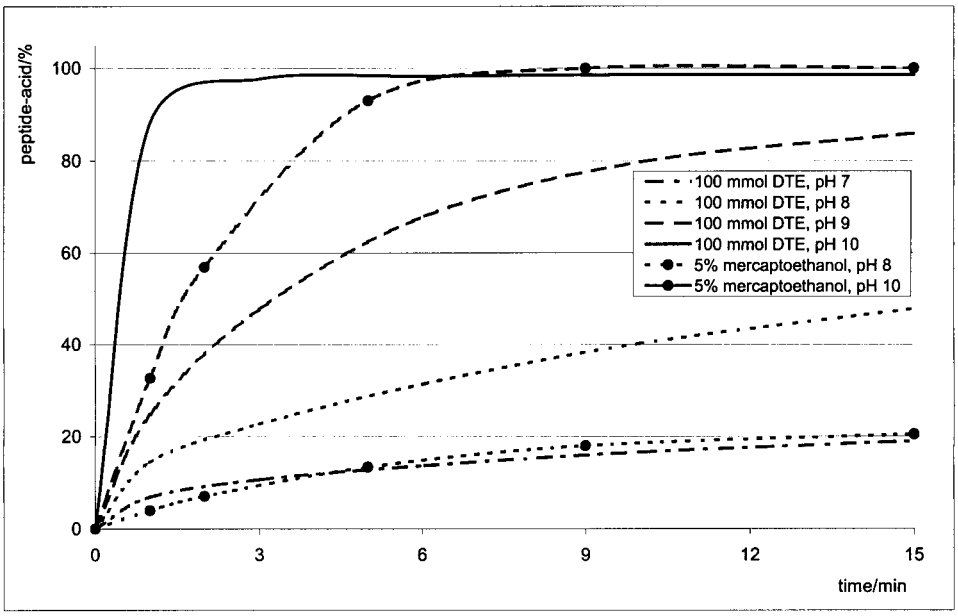


Fig. 1. *pH Dependence of the conversion* **7** → **10** *at constant concentrations of both mercaptoethanol and DTE*

To investigate whether reductive hydrolysis is applicable to single-bead analysis, ten different peptides (*Table 1*) were synthesized on PEGA resin carrying the above disulfide linker. In all cases, quantitative cleavage was observed, and the peptides were analyzed by tandem MS as shown for Phe-Pro-Met-Lys-Thr (*Fig. 2*). Ionization of doubly charged species preferentially yields the y-fragments (above), while ionization of the molecular peak yields characteristic b-fragments (below). These fragments, together with those corresponding to single amino acids, allowed us to determine the exact peptide sequence as shown in *Table 2*.

Table 1. *MS-Analysis of Various Peptides Synthesized on PEGA Beads and Cleaved with 2-Mercaptoethanol*

Sequence	Cleavage with 2-mercaptoethanol	
	calculated $[M+H]^+$	measured $[M+H]^+$
Gln-Arg-Pro-Tyr-Gly	620	620
Phe-Pro-Met-Lys-Thr	623	623, 639
Cys-Arg-Asn-Asp-Cys	608	[a])
Trp-Tyr-Ser-His-Glu	721	721
Met-Phe-Gln-Gly-Ile	595	595
Phe-Leu-Ser-Val-Arg	621	621
Gly-Ala-Arg-Cys-Phe	553	[a])
Cys-Gly-Ala-Arg-Cys-Phe	656	[a])
Ile-Gly-Glu-Pro-His	552	552
Ala-Asn-Ile-Gly-Glu-Pro-His	737	737

[a]) No product observed.

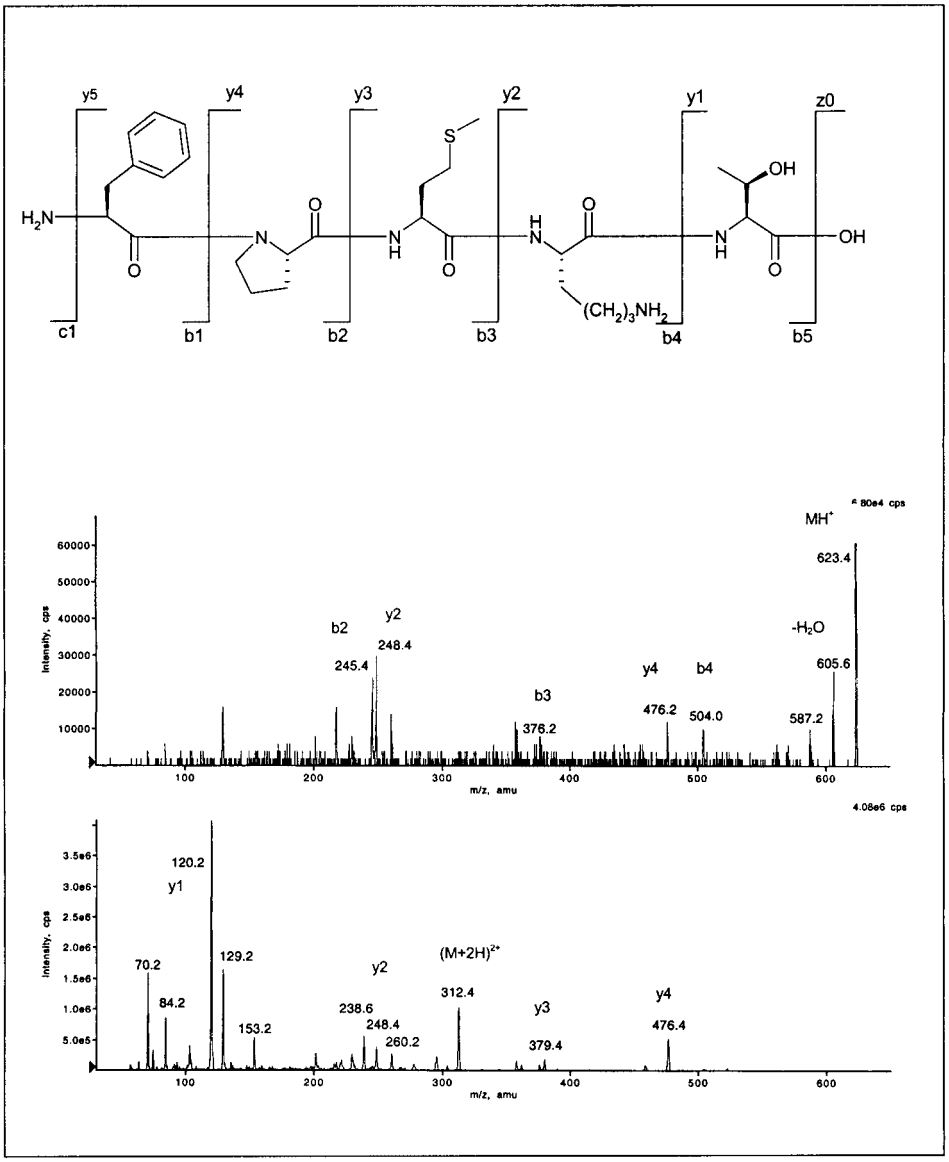


Fig. 2. *Characteristic fragmentation of Phe-Pro-Met-Lys-Thr and its MS/MS from single- and double-charged molecular ions*

Conclusions. – We have developed a suitable disulfide-linker for solid-phase peptide synthesis and subsequent MS analysis. The synthesis of the PEGA-resin with a functional loading of 0.17 mmol/g is straightforward, and the linker survives the usual conditions of peptide synthesis and deprotection. Most importantly, peptide cleavage can be achieved from a single bead within 2 min to obtain a sample ready for

Table 2. *MS Fragments of Peptides from Single-Bead Analysis*

Sequence	Molecular peak	b-Fragments				y-Fragments				
		1	2	3	4	1	2	3	4	5
Gln-Arg-Pro-Tyr-Gly	620		285					239	336	492/475
Phe-Pro-Met-Lys-Thr	639 (ox)		245	376	504	120	248	379	476	
Trp-Tyr-Ser-His-Glu	720						285	372	535	
Met-Phe-Gln-Gly-Ile	595	132	279		464/480	143	189	317	464	
Phe-Leu-Ser-Val-Arg	621	261				175	274	361	474	
Ile-Gly-Glu-Pro-His	552	114	186	299	397	156	253	380	439	535
Ala-Asn-Ile-Gly-Glu-Pro-His	737		186	299			439	552		721

sequencing by tandem MS. Accordingly, this method is suitable for fast analysis of, *e.g.*, 'split & mix libraries' of short peptides.

Experimental Part

General. For all syntheses, PEGA-resin (PL-PEGA, 0.2 mol-equiv./g, *Polymer Labs.*) modified with *N*-methylglycine (sarcosine) was used. All solvents and reagents were commercially available and used without further purifications. UV/VIS Spectra: *Kontron Instruments UVIKON 860*. HPLC Analyses: *Hewlett-Packard HPLC1050* with *YMC-Pack ProC18*, *AS-300* column. IR Spectra (cm^{-1}): *Nicolet 20 SXB FTIR* and *Nicolet 550* for analyses of single beads. NMR Spectra: *Bruker 400* (if not stated otherwise) with $SiMe_4$ as internal standard at 300 K. MS Spectra: *Perkin-Elmer Sciex API III*.

2-({2-[1,1-Bis(4-methoxyphenyl)-1-phenylmethoxy]ethyl}disulfanyl)ethanol (**1**). To a soln. of 2-[(2-hydroxyethyl)disulfanyl]ethanol (9.5 ml, 78 mmol) in anh. pyridine (5 ml), a soln. of 4.4′-Dimethoxytriphenylmethyl chloride (20.5 g, 60 mmol) in anh. pyridine (100 ml) was added within 2 h under stirring at r.t. The mixture was stirred for 16 h under N_2, quenched within 30 min with MeOH (20 ml), poured on H_2O (50 ml), and extracted with CH_2Cl_2. The combined org. layers were washed with sat. aq. NaCl soln., dried ($MgSO_4$), and co-evaporated azeotropically with toluene to yield a dark yellow oil. The residue was purified by CC (SiO_2; hexane/*t*-BuOMe 1:1 containing 2% of Et_3N) to furnish **1** as a highly viscous yellow oil (15 g, 55%). IR (KBr): 3432, 1736, 1608, 1445, 1251, 1070. ^{1}H-NMR (250 MHz, $(CD_3)_2SO$): 2.68 (*t*, 2 H, $J = 6.48$); 3.53–3.58 (*m*, 2 H, $J = 6.2, 5.8$); 2.91 (*t*, 3 H, $J = 6.01$); 3.21 (*t*, 2 H, $J = 6.09$); 3.73 (*s*, 6 H); 4.84 (*t*, 1 H, $J = 5.4$); 6.8–7.4 (*m*, 13 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 41.4, 46.12, 55.4, 59.84, 62.07, 86.04, 113.56, 127.0–128.2, 130.03, 136.04, 145.25, 158.49. ESI-MS: 456 $(M)^+$, 379 ($[M - C_6H_5]^+$), 303 (DMT^+)[4]. ISP-MS: 303 (DMT^+). ISN-MS: 515 ($[M + Ni]^+$). Anal. calc. for $C_{25}H_{28}O_4S_2$: C 65.76, H 6.18, S 14.04; found: C 65.93, H 6.49, S 13.17.

13-Bis(4-methoxyphenyl)-13-phenyl-4-oxo-8,9-dithia-5,12-dioxatridecanoic Acid (**2**). Alcohol **1** (15 g, 33 mmol), succinic anhydride (10.1 g, 90 mmol), and DMAP[5]) (12 g, 99 mmol) were dissolved in anh. pyridine (120 ml) and CH_2Cl_2 (120 ml) at r.t. The mixture was stirred for 24 h under N_2, poured on 1% aq. acetic acid soln. (500 ml), and extracted with CH_2Cl_2. The combined org. layers were washed with sat. aq. NaCl soln., dried over $MgSO_4$, and evaporated. The remaining pyridine was co-evaporated with toluene, leaving a brown oily residue, which was purified by CC (SiO_2; MeOH/AcOEt 1:9 containing 2% of Et_3N) to yield **2** (12.1 g, 66%) as a highly viscous yellowish oil. IR (KBr): 2496, 1737, 1608, 1445, 1251, 1157, 1170, 1034, 832. ^{1}H-NMR ($(CD_3)_2SO$): 2.6 (*m*, 4 H); 2.79 (*t*, $J = 6.4$, 2 H); 2.86 (*t*, $J = 6.4$, 2 H); 3.36 (*t*, $J = 6.4$, 2 H); 3.79 (*s*, 6 H); 4.28 (*t*, $J = 6.6$, 2 H); 6.9, 7.2–7.4 (*m*, 13 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 41.4, 55.4, 59.84, 86.04, 113.52, 127.0–128.2, 130.45, 136.04, 145.25, 158.49. ISN-MS: 555 ($[M - H]^+$). Anal. calc. for $C_{29}H_{32}O_7S_2$: C 62.57, H 5.79, S 11.52; found: C 61.45, H 6.14, S 9.29.

Coupling of **2** *to the PEGA resin*. To 700 mg of PEGA resin (*ca.* 0.14 mmol, 4.8 g in MeOH), rinsed with NMP[6]), a preactivated linker-soln. in NMP was added, consisting of 950 µl of a 0.5M (0.42 mmol) soln. of

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[4]) DMT represents the [1,1-Bis(4-methoxyphenyl)-1-phenyl]methyl group.

[5]) DMAP represents 4-(dimethylamino)pyridine.

[6]) NMP stands for *N*-methyl-2-pyrrolidon.

HATU[7]), 430 µl of a 1M (0.42 mmol) soln. of HOAt[8]), 470 µl (0.85 mmol) Et(i-Pr$_2$)N, and 230 mg (0.42 mmol) of **2**. The mixture was diluted with NMP (half the volume of the resin) and shaken for 16 h. The beads were washed for 2 min each with NMP (2×) and i-PrOH (2×), and this procedure was repeated once. The loading varied between 0.1 mmol/g and 0.17 mmol/g. Anal. of **2** on PEGA resin: C 62.26, H 6.27, S 11.08. Anal. of PEGA-resin: C 48.9, H 7.90. IR (KBr): 1720, 1547.

Cleavage of the trityl-derived Protecting Group (**3** → **4**). The linker-containing PEGA resin was treated for 30 min with a soln. of $CF_3CO_2H/H_2O/(i\text{-}Pr)_3SiH$ (95 : 3 : 2) and then washed alternately with NMP and i-PrOH. IR (KBr): 1201 (OH). Anal. of **4** on PEGA-resin: C 58.16, H 6.30, N 2.95, S 13.5. The epitopic density was controlled by measuring the UV-absorption of DMT-cation[4]) in $HClO_4$/EtOH 3 : 2 at $\lambda = 499$ nm ($\varepsilon = 71700$).

Coupling of the First Amino Acid to the Modified Resin (**4** → **5**). To 650 mg of the modified resin **4** (0.13 mmol), rinsed with NMP, were added 430 µl (0.19 mmol) of a 0.5M amino acid soln. (Fmoc-Gly) in NMP, 39 mg (0.19 mmol) of dicyclohexylcarbodiimide (DCC), and 15 µl of a 13.5 mM DMAP soln. in anh. pyridine (1.9 µm DMAP and 0.19 mmol pyridine). The mixture was diluted with CH_2Cl_2 (50% of the volume of the resin) and was shaken for 3 h. The resin was washed as described above, and the coupling procedure was repeated under the same conditions. The coupling of the first amino acid runs to completion as determined by UV upon removing the Fmcoc group ($\varepsilon_{300} = 7840$), see below. Anal. of **5** on PEGA resin: C 48.5, H 5.9, S 16.9.

Peptide Synthesis by Fmoc-Protocol. A soln. consisting of 5 equiv. of Fmoc-protected amino acid (0.5M soln. in NMP), 5 equiv. of HATU (0.5M soln. in NMP), 5 equiv. of HOAt (1M soln. in NMP), and 10 equiv. of Et(i-Pr)$_2$N was added to one equiv. of deprotected resin. The mixture was diluted with NMP (50% of the volume of the resin) and shaken for 1.5 h. The resin was washed as described above with NMP and i-PrOH. The beads were treated with a 20% soln. of piperidine in NMP to cleave the terminal Fmoc group and washed again. At the end of peptide synthesis, the resin was treated with a soln. of $CF_3CO_2H/(i\text{-}Pr)_3SiH/H_2O$ 95 : 3 : 2 to deprotect the side-chain protecting groups.

Single-Bead Analysis of Peptides. Under a microscope, a single bead was selected and transferred into a melting-point tube. Approx. 6 µl of a 100 mM soln. of mercaptoethanol in MeCN (containing 0.18% of NH_4OH) were added, and the tube was centrifuged shortly to ensure that the bead was suspended. The reaction was quenched by adding 5 µl of 40% aq. HCO_2H soln. Such samples can be stored for several days without decomposition. Immediately before analysis, the soln. was purified over *Zip Tip* pipette tips (*Millipore*) [12] and diluted with 5% aq. HCO_2H soln. to a volume of 100 µl. The aspirated sample was bound onto the chromatographic media. With 5 µl of a 5% HCO_2 soln. in 60% of aq. MeCN the peptide was eluted, ready for MS.

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7) HATU stands for *N,N,N′,N′*-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate.
8) An aza-analog of hydroxybenzotriazol: 3*H*-[1,2,3]triazolo[4,5-*b*]pyridin-3-ol.