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A new water soluble 3,6,9-trioxaundecanedioic acid-based linker and biotinylating reagent

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ABSTRACT

Biotinylated peptides often have low solubility in water. In this Letter, we describe a new method to synthesize a biotinylating reagent for water-solubilizing hydrophobic peptides. The biotinyl-6-aminohexanoic derivatives prepared contain a hydrophilic 3,6,9-trioxaundecanedioic acid linker moiety between the biotin and the peptide to improve the water solubility, and also to function as a spacer. The monoesterified derivative of 3,6,9-trioxaundecanedioic acid was synthesized, and the Fmoc-protected ethylenediamine was used to link to the carboxylic group of biotin. The hydrophilic nature of this new biotin–peptide conjugate was also demonstrated in a comparative analysis of compounds containing a biotinyl-6-aminohexanoic acid or 3,6,9-trioxaundecanedioic acid derivative.

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Biological measurements of peptides with low solubility can cause problems in their evaluation. Biotin-labeled peptides have even lower solubility than unlabeled ones. Such labeled peptides are widely applied in in vitro binding assays because of the strong binding of biotin to the proteins avidin and strepavidin.[1–5] In many cases, to avoid steric hindrance, the even less soluble biotinyl-6-aminohexanoic acid is used[6,7] in the high affinity streptavidin–biotin binding system, which is widely utilized in clinical diagnostic applications,[8] but which causes further solubility difficulties. To overcome this problem several approaches have been described in the literature that are based on oligoethyleneglycol linkers with known molecular mass; in these studies mainly 4,7,10-trioxa-1,13-tridecanediamine[9–11] was used.

The primary goal of our research was to develop a new 3,6,9-trioxaundecanedioic acid-based biotin-spacer analog, which is more soluble than biotinyl-6-aminohexanoic acid. The novelty of our approach is the inclusion of 3,6,9-trioxaundecanedioic acid (TEG) modified with ethylenediamine (EDA) as a soluble linker between the biotin and the oligopeptide. Our design requires protected building blocks to create the biotinylating reagent via solid-phase step-wise synthesis. We describe herein the synthesis of the new biotinyl-EDA–TEG biotinylating reagent outlined in Figure 1 with the ability to solubilize the hydrophobic oligopeptide that it is attached to. The reagent was utilized for labeling the synthetic hydrophobic model peptide-amide, NH_2-Glu-Val-Thr-Cys(Acm)-Val-Val-Val-Asp-NH_2 (*ED*) which has low water solubility.[12] To study the efficacy of the linker moiety to solubilize peptides, we describe a comparison of the water solubility of the *ED* peptide and that of its biotinylated derivatives using biotin, biotinyl-6-aminohexanoic acid, and the 3,6,9-trioxaundecanedioic acid-based biotin–EDA–TEG, and also that of the unbiotinylated, but EDA–TEG-labeled *ED* peptide as a control.

As both EDA and TEG building blocks are bifunctional, in order to perform chemoselective coupling reactions we required their mono-protected derivatives. We used the butyl ester form of 3,6,9-trioxaundecanedioic acid (TEG-OBu) and Fmoc-protected ethylenediamine (Fmoc–EDA). The dicarboxylic acid was transesterified with butyl formate using Dowex ion-exchange resin in octane.[13,14] Within the Dowex resin a strongly acidic water layer is formed. A partition equilibrium between the aqueous layer and the aprotic butyl formate/octane layer is established, and 3,6,9-trioxaundecanedioic acid has a higher partition coefficient for water

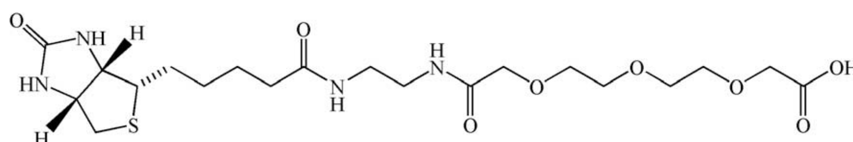

Figure 1. Structure of the new biotinylating reagent based on 3,6,9-trioxaundecanedioic acid modified with ethylenediamine.

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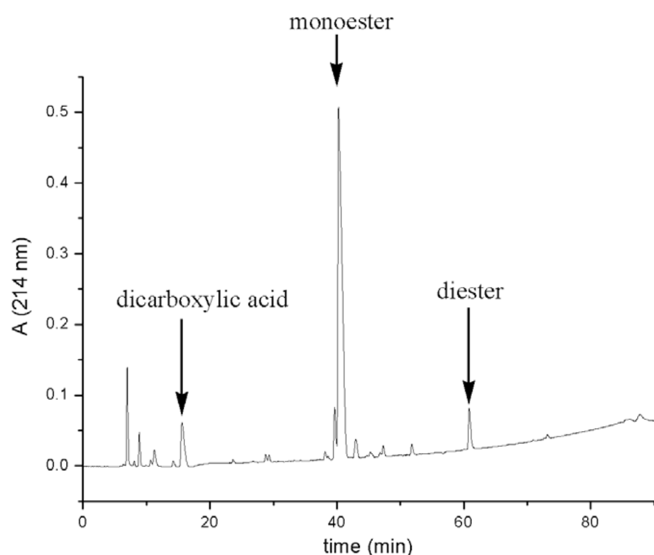
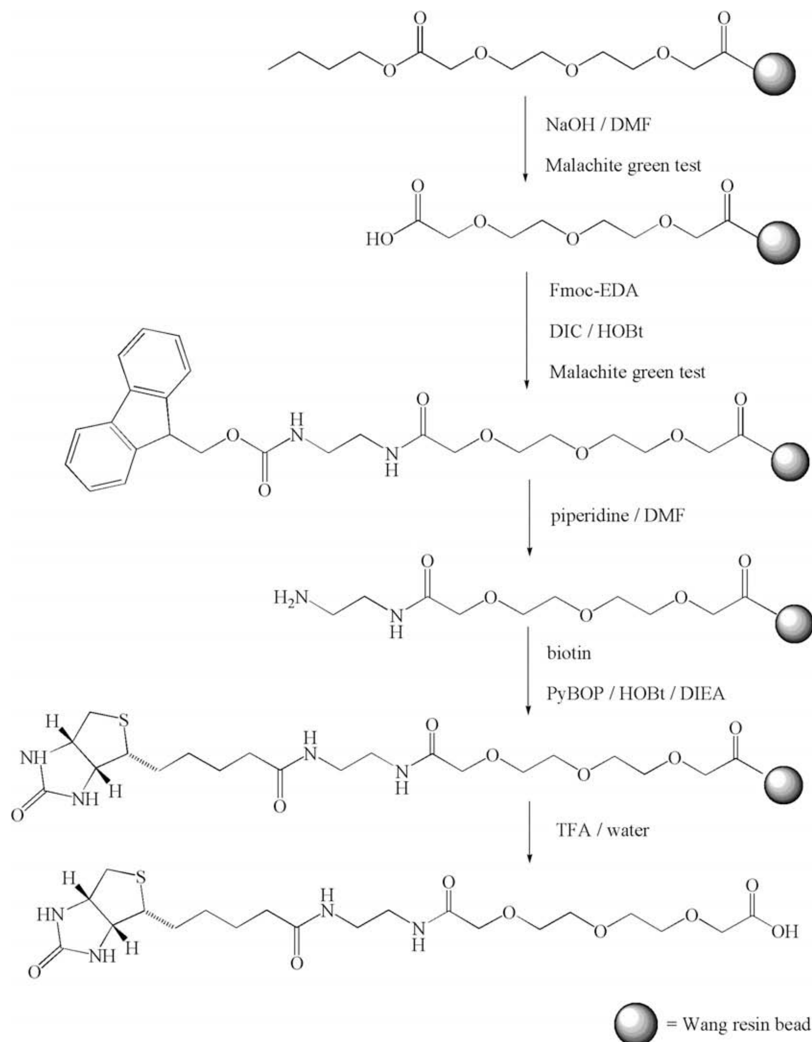


Figure 2. RP-HPLC chromatogram of the crude monoester of 3,6,9-trioxaundecanedioic acid. Eluent A: water/0.1% TFA, eluent B: MeCN:water/4:1/0.1% TFA, flow: 1 mL/min, gradient: 5 min—5% B; 90 min—95% B.

than the product monobutyl ester. Esterification of the carboxylic acid takes place in the aqueous layer and/or at the interface between the aqueous and the non-aqueous layers. The resulting monoester moves from the aqueous layer into the aprotic layer and remains there without reacting further. Figure 2 shows the RP-HPLC chromatogram of the crude reaction mixture after completion of the reaction, the major product of which is the monobutyl ester of 3,6,9-trioxaundecanedioic acid.

The biotinyl-EDA–TEG was synthesized on solid phase (Scheme 1).[15] The monoprotected TEG-butyl ester was coupled to Wang resin and the butyl-protecting group was removed under basic conditions. We monitored the success of the cleavage via the Malachite green test.[16] By forming a green salt with a carboxylic acid, Malachite green can indicate the presence of a free carboxylic group, thus showing whether the ester group has hydrolyzed. Next, to couple biotin and TEG, both having carboxylic groups, we used ethylenediamine. Fmoc–ethylenediamine was coupled to the resin-attached-TEG with DIC/HOBt. The success of the coupling could be checked again with the Malachite green assay, because after completion of the reaction, the resin beads do not turn green. Unlike conventional solid-phase peptide synthesis, we create an 'inverse' amide bond by coupling the free amino group-containing compound to the free carboxylic group on the resin. After Fmoc deprotection with piperidine we were able to biotinylate the free amino group using the PyBOP/HOBt coupling method.



Scheme 1. Synthesis of the new biotinylating reagent based on 3,6,9-trioxaundecanedioic acid modified with ethylenediamine (EDA–TEG).

Table 1
Characteristics of the *ED* peptide and *ED*-peptide conjugates

Peptides and conjugates	t_R[a] (min)	$[M+H]^+_{calcd}$	$[M+H]^+_{found}$[b]	Solubility at 25 °C[c] (mg/mL H_2O)
EVTC(Acm)VVVD (*ED*)	17.41	933.1	933.6	1.6
Biotinyl-*ED*	26.39	1159.4	1159.6	0.6
Biotinyl-6-aminohexanoic amidyl-*ED*	30.53	1272.6	1272.7	0.4
EDA–TEG-*ED*	26.41	1179.4	1179.6	1.9
Biotinyl-EDA–TEG-*ED*	28.33	1405.7	1405.7	1.0

[a] Column: Phenomenex Synergi MAX-RP C12 (4 μm, 4.6 mm × 25 cm, Torrance, CA, USA). Isocratic elution with 15% eluent B was applied for 5 min, then a linear gradient from 5% to 95% eluent B was generated over 30 min at room temperature (eluent A: 0.1% TFA in water and eluent B: 0.1% TFA in acetonitrile–water (80:20, v/v). Flow rate: 1 mL/min. Peaks were detected at λ = 214 nm.

[b] Bruker Esquire ESI-MS. Masses presented as $[M+H]^+$ were calculated from multiple charged ions.

[c] 30 mg of purified compound was dissolved in 1.2 mL of distilled water. After 24 h the sample was centrifuged (6000 rpm, 25 min) and 1 mL of the supernatant was freeze dried and stored under vacuum over P_2O_5 until a constant weight was attained.

The synthesis of the biotinyl-EDA–TEG-*ED* peptide conjugate was performed manually on solid phase. First the peptide chain of *ED* was assembled by automated solid-phase methodology using Rink–Amide–MBHA resin. All amino acids were coupled as Fmoc-derivatives. The side chain of the Cys residue was protected with an *S*-acetamidomethyl group (Acm), which is stable both under acidic and alkaline conditions. Activation and coupling were carried out using DIC/HOBt in DMF. Fmoc groups were removed with piperidine in DMF. After cleavage of the Fmoc-protecting group from the terminal N^{α}-amino function the resin-bound *ED* peptide was acylated with biotinylating reagents (biotin, biotinyl-6-amino-hexanoic acid, and biotinyl-EDA–TEG) using a threefold excess in the presence of a threefold excess of DIC/HOBt in DMF. We also prepared the biotinyl-EDA–TEG-*ED* conjugate by an alternative route. We generated the Fmoc–EDA–TEG linker moiety in solution phase[17] and attached it to the N-terminus of the resin-bound peptide. After removal of the Fmoc-protecting group in the second step, we attached biotin to the amino group of the EDA–TEG-*ED*-resin (ninhydrin assay monitoring). The conjugate was cleaved from the resin with TFA/water, purified by RP-HPLC, and characterized by mass spectrometry. Unbiotinylated EDA–TEG-*ED* was also cleaved, isolated, and characterized. The HPLC retention time values and the ESI mass spectrometry data of the labeled peptide conjugates are summarized in Table 1.

A solubility study of these peptide conjugates was performed in water as described by Malavolta and Nakaie,[18] and these data are presented in Table 1. The attachment of biotin to the *ED* peptide reduced the solubility of the peptide conjugate by 62.5%, and attachment of biotinyl-6-aminohexanoic acid reduced the solubility by 75%. Comparison of the water solubility of biotinylated *ED* peptide with different biotinylating moieties shows significant differences: the solubility of the biotinylated peptide was 1.5 times higher than that of the peptide possessing biotinyl-6-aminohexanoic-amide (0.6 vs 0.4 mg/mL). Insertion of the EDA–TEG moiety between the biotin and the peptide markedly improved the solubility (0.6 mg/mL for biotin-*ED* and 1.0 mg/mL for biotinyl-EDA–TEG-*ED*). Attachment of EDA–TEG to the peptide without the biotin moiety slightly enhanced the solubility of the *ED* peptide (18%).

In conclusion, we have synthesized a new, 3,6,9-trioxaundecanedioic acid-containing biotinylating reagent, biotinyl-EDA–TEG, by a step-wise synthesis to afford water-soluble biotin-labeled peptide conjugates. During the synthesis of biotinyl-EDA–TEG, unlike conventional solid-phase peptide synthesis, we created an 'inverse' amide bond by coupling the free amino group-containing compound to the free carboxylic group on the resin. The success of the coupling was monitored by Malachite green assay. We have also prepared an Fmoc-protected oligoethyleneglycol (Fmoc–EDA–TEG) spacer molecule in solution phase using common coupling reagents and solvents. Coupling of the EDA–TEG linker and biotin to the hydrophobic model peptide EVTC(Acm)VVVD proved to be efficient in improving the water solubility compared to that of biotinyl-6-aminohexanoic acid (1.0 mg/mL vs 0.4 mg/mL, respectively), and could be useful for the biotinylation of peptides and facilitation of the use of hydrophobic peptides in avidin/strepavidin-based binding assays.

Acknowledgment

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14. *Synthesis of the monobutyl ester of 3,6,9-trioxaundecanedioic acid*: 3,6,9-trioxaundecanedioic acid (1 g, 45 mmol) was refluxed with 5 g of Dowex X-5 ion-exchange resin, in 200 mL of butyl formate/octane mixture (1:1 v/v%) for 3 h. Then the resin was filtered and the solvent was evaporated in vacuo. The product was obtained as a colorless oil in about 90% yield and was used without any further purification. MS: $[M+H]^+_{found}$: 249.6 ($[M+H]^+_{calcd}$: 250.3).
15. The synthesis was performed on Wang resin (1 mmol/g). The 3,6,9-trioxaundecanedioic acid butyl ester (667 mg, 3 mmol) was coupled to 1 g of resin using DIC (465 μL, 3 mmol) and DMAP (37 mg, 0.3 mmol) in DMF for 3 h. The butyl ester-protecting group was removed with 20% 1 M NaOH/DMF mixture in 30 min. The success of the butyl ester cleavage was checked using the Malachite green indicator. The product was activated with DIC (465 μL, 3 mmol) and HOBt (405 mg, 3 mmol) in 5 mL of DMF for 15 min, and Fmoc-EDA (480 mg, 3 mmol) was added to 5 mL of DMF and reacted for 2 h. The success of the coupling was checked using the Malachite green test. The Fmoc group was removed with 20% piperidine/DMF (2 + 2 + 5 + 20 min). Biotin (732 mg, 3 mmol) was coupled using PyBOP (1.56 g, 3 mmol) and HOBt (405 mg, 3 mmol)/DIEA (1.7 mL, 10 mmol). The success of the coupling was checked with the ninhydrin assay. The product was cleaved from the resin with TFA/water 9.5:0.5 mL for 1.5 h at 0 °C. After filtering off the resin, cold diisopropyl ether was added, and the precipitated product was separated by filtration. The product was purified by RP-HPLC, and was characterized by MS. MS: $[M+H]^+_{found}$: 491.2 ($[M+H]^+_{calcd}$: 491.6).
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