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Stable Attachment of the HMB-Linker to Continuous Cellulose Membranes for Parallel Solid Phase Spot Synthesis

Rudolf Volkmer-Engert, Berit Hoffmann and Jens Schneider-Mergener

Institut für Medizinische Immunologie, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, Schumannstr. 20-21, D-10098 Berlin, Germany

Abstract: Peptides and other oligomeric molecules were prepared on continuous cellulose membranes using a stably attached p-hydroxymethyl-benzoic acid (HMB)[1] linker. After attachment of N-protected 1,2-epoxy-propylamine to the support via an ether bond, the HMB linker was coupled to this construct resulting in a orthogonal safety-catch linkage suitable for solid phase synthesis. As an application, several peptides, peptoids and carbohydrate-peptide conjugates were prepared and analyzed by HPLC and mass spectrometry. © 1997, Elsevier Science Ltd. All rights reserved.

Solid phase synthesis on cellulose supports[2], in particular the spot synthesis technique[3], has become an efficient and inexpensive tool for the rapid preparation of large numbers of membrane-bound peptides and peptide libraries useful for the identification of single molecules that bind given ligands, such as proteins, metals and nucleic acids[4-8]. However, there is a need for testing large numbers of soluble peptides and other compounds directly in biological test systems. We developed and evaluated a novel strategy for the parallel solid phase spot synthesis on continuous cellulose membranes using the HMB linker[9], which is stably attached to the support via an ether bond. This construct allows the stepwise synthesis and subsequent simple cleavage of the products from the membrane using ammonia vapour[10]. Several peptides, peptoids and carbohydrate-peptide conjugates were synthesized, deprotected, released and characterized.

The attachment of the HMB linker (Fig. 1) was achieved by: (i) Lewis-acid catalyzed ring-opening of the N-protected epoxypropylamine by the cellulose hydroxyl groups[12]. Two epoxides were used: N-(2,3-epoxypropyl)phthalimide (**1**) and 2,3-epoxy-N-(9-fluorenylmethoxycarbonyl)-propylamine[11] (**2**). (ii) Cleavage of the Fmoc- or Phth-protecting group[12] resulting the modified cellulose membrane **3**. (iii) N-acylation of the amino-hydroxypropyl cellulose ether **3** with p-hydroxymethylbenzoic acid (TBTU/DIEA activation[13], 1.2 µl of a 0.3 molar solution of HMB in DMF containing 1.1 eq. TBTU and 2 eq. DIEA for each spot, double coupling, 15 min each). (iv) Attachment of the first amino acid (DIC/NMI activation[3], 1.2 µl of a 0.3 molar solution of N-α-Fmoc-O-t-butyl-L-threonine in DMF containing 1.1 eq. DIC and 2 eq. NMI, 2 h reaction time, tree times repeated) and cleavage of the Fmoc protecting group[14] resulted the modified cellulose membrane **5**.

In a second approach the preformed N-α-Fmoc-glycine-(4-hydroxy-methylbenzoic acid)-ester **4**[15] was

used as acylating reagent (membrane **3**, HATU/DIEA activation[16], 1.2 µl of a 0.3 molar solution of **4** in DMF containing 1.1 eq. HATU and 2 eq. DIEA for each spot, double coupling, 15 min each). Cleavage of the Fmoc protecting group[14] yielded in the modified cellulose membranes **6**.

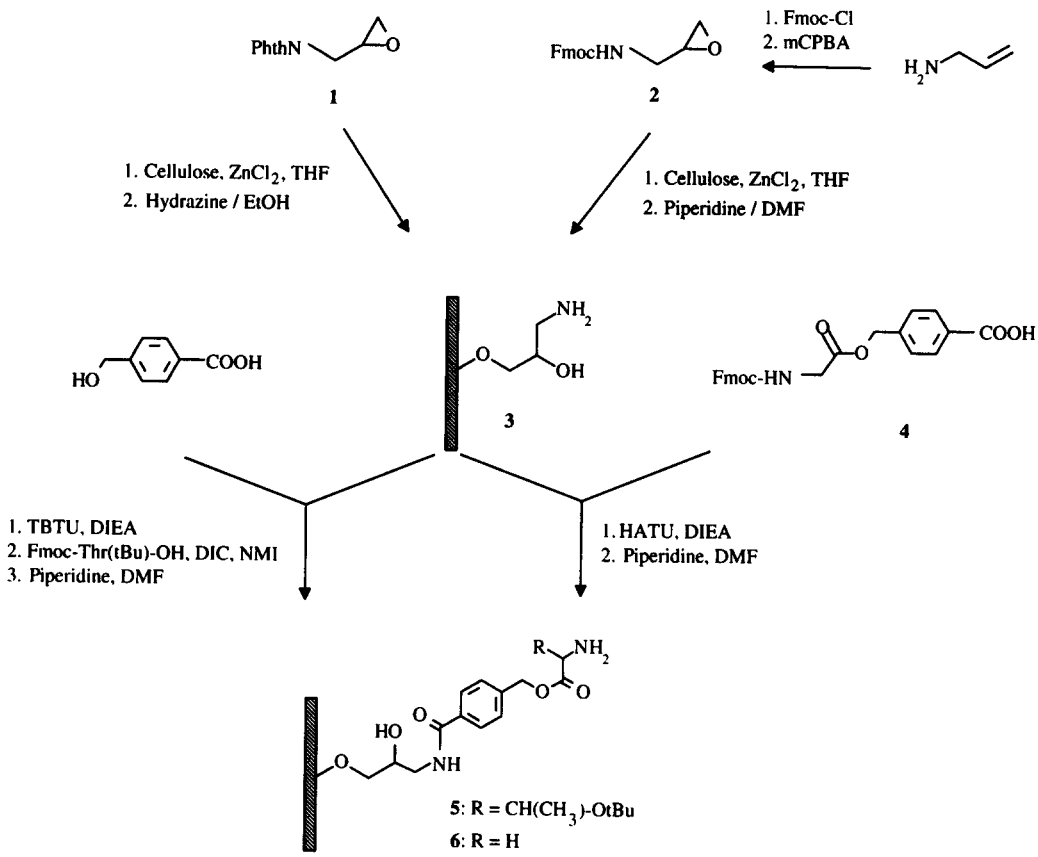
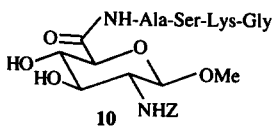
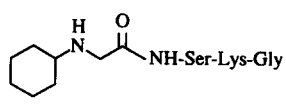


Figure 1: Synthetic scheme for the attachment of the HMB linker to the cellulose membrane

Attachment of the Fmoc-epoxy compound **2** to the membrane was quantified by measuring the absorbance of the released Fmoc-group (ε_{302} = 8100, 70 - 85 nmol/cm^2). The N-Fmoc-epoxy derivative **2** proved to be more useful due to milder deprotection conditions. According to the spot synthesis protocol[3], the peptides **7** (GATPQDLNT), **8** (VVSHFNDG) and **9** (GATPQDLNTG) were assembled on the modified cellulose membranes. Moreover, the carbohydrate-peptide conjugate **10** as well as the peptoid **11** were prepared.





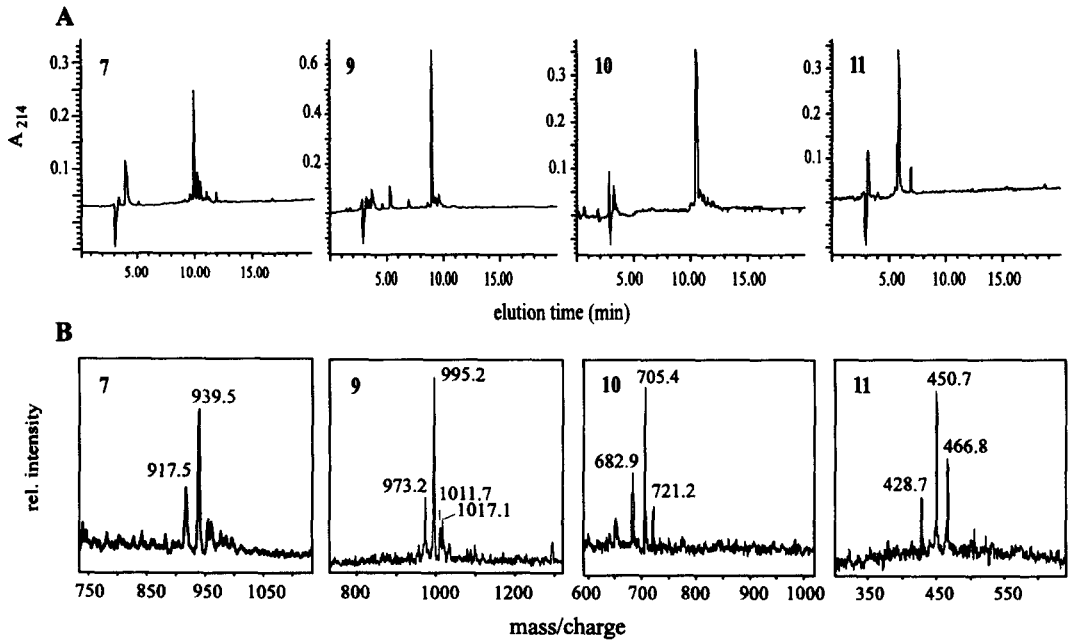


Figure 2: RP-HPLC profiles (**A**) and MALDI-TOF mass spectra (**B**) of the crude products (**7, 9-11**) synthesized on **5** or **6**. HPLC: linear gradient 5%-40% acetonitrile/water (0.05% TFA) for 20 min at 1.2 ml/min flow rate on a RP-C_{18} column. MALDI-TOF: matrix: α-cyano-cinamic-acid (see also Tab. 1).

Compound **10** was prepared by acylating (TBTU/DIEA activation[13]) the membrane bound tripeptide (SKG) with the aminouronic acid-alanine conjugate[17]. Acylating the same peptide with bromoacetic acid (1.2 µl of a 0.3 molar solution containing 1.1 eq. DIC), followed by nucleophilic displacement of the halogen by cyclohexylamine, let to the peptoid **11**. Three equivalents cyclohexylamine (double coupling, 15 min) were used. After cleavage of the side chain protection groups[3,6], (TFA/DCM 1:1, 3% triisobutylsilane, 2% water) the compounds **7-11** were released as carboxamides by treating the cellulose membrane with ammonia vapour[10] for 5 hours[18]. The spots were punched out and transferred to wells of microtiter plates. Subsequently the adsorbed compounds were extracted with water (160 µl/spot) and analyzed by HPLC and MALDI-TOF mass spectrometry (Fig. 2). The amount of peptide received from one spot (0.32 cm^2) was determined by HPLC (Tab. 1). Using the modified cellulose membrane **6**, up to 24 nmol peptide/spot were obtained. In contrast, a significantly smaller amount of 13 nmol peptide/spot was recovered using the modified cellulose membrane **5**.

In conclusion, this approach can be used for the preparation of soluble peptide sets as well as combinatorial peptide and organic libraries automatically prepared on continuous cellulose membranes[19]. After transfer to microtiter plates, the compounds can be released and directly used in biological screening systems.

Table 1: Characterization and quantification of the crude compounds **7-11** synthesized on the modified cellulose membrane **5** and **6**.

Compound No.	Molecular Mass calc.[M]	Molecular Mass observed Mass	Quantity [nmol/spot][a)]
7	916.0	917.5 = $[M+H]^{\oplus}$, 939.5 = $[M+Na]^{\oplus}$	13
8	872.9	873.5 = $[M+H]^{\oplus}$, 895.7 = $[M+Na]^{\oplus}$, 911.8 = $[M+K]^{\oplus}$	21
9	972.0	973.2 = $[M+H]^{\oplus}$, 995.2 = $[M+Na]^{\oplus}$, 1011.7 = $[M+K]^{\oplus}$	24
10	683.7	682.9 = $[M+H]^{\oplus}$, 705.4 = $[M+Na]^{\oplus}$, 721.2 = $[M+K]^{\oplus}$	n. d.
11	428.5	428.7 = $[M+H]^{\oplus}$, 450.7 = $[M+Na]^{\oplus}$, 466.8 = $[M+K]^{\oplus}$	n. d.

a) area: 0.32 cm^2

References and Notes

1. Special abbreviations used: HMB: hydroxymethylbenzoic acid; MCPBA: 3-chloroperoxybenzoic acid; Phth: phthaloyl; Fmoc: 9-fluorenylmethoxycarbonyl-; TBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIEA: diisopropylethylamine; DIC: diisopropylcarbodiimide; TFA: trifluoroacetic acid; DCM: dichloromethane; NMI: N-methyl-imidazole; HATU: O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.
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11. Synthetic procedure for **2**: 5.8 g (0.1 mol) allylamine and 15.2 ml (0.11 mol) triethylamine were dissolved in 300 ml dichloromethane at 0^0C. 28.4 g (0.11 mol) 9-fluorenylmethyl chloroformate dissolved in 1000 ml dichloromethane was added dropwise under stirring. After additional 4 h at RT the reaction mixture was washed with diluted hydrochloric acid, water and dried ($MgSO_4$). Removal of the solvent and crystallization from ethanol yielded 20.2 g (72%) N-(9-fluorenylmethoxycarbonyl)allylamine. m.p. 121^0C.
    5.6 g (20 mmol) N-(9-fluorenylmethoxycarbonyl)allylamine was dissolved in 200 ml acetone and 40 ml water. 8.8 g (50 mmol) 3-chloroperoxybenzoic acid was added in portions and the mixture was heated to reflux for additional 12 h. After careful removal of the organic solvent, keeping the volume nearly constant by adding water, the aqueous solution was extracted with dichloromethane. The extract was washed with aqueous sodium hydrogen carbonate and water, dried ($MgSO_4$) and evaporated. Chromatography on Silica Gel (Si60, 40-63µm) with 1:1 hexane-ethyl acetate gave 2,3-epoxy-N-(9-fluorenylmethoxycarbonyl)propylamine (**2**) (4.4 g, 76%). m.p. 118^0C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.2 - 7.8 (m, 8H, fluorenyl-), 4.9 (m, 1H, NH), 4.4 (dd, 2H, $-CH_2OCO-$), 4.2 (t, 1H, -CH-), 3.6 (m, 1H, $-CH_2$), 3.3 (m, 1H, $-CH_2$), 3.1 (m, 1H, -CHO-), 2,7 (m, 1H, $-CH_2O-$), 2.5 (m, 1H, $-CH_2O-$). MS (EI,150^0C): m/z = 295 [$M^{\oplus}$].
12. Synthetic procedure for **3**: Whatman 50 paper (10 x 7 cm) was washed in a stainless steel dish with 20 ml 25% trifluoroacetic acid in dichloromethane for 20 min, 2 x 20 ml dichloromethane, 2 x 20 ml 10% triethylamine in dichloromethane, 2 x 20 ml dichloromethane, 2 x 20 ml tetrahydrofuran and dried over phosphorus pentoxide. In a closed stainless steel dish the dried paper sheet was soaked with a solution of **1** or **2** (6.1 mmol) and zinc chloride (8.8 mmol) in abs. tetrahydrofuran. After 24 h the membrane was washed with tetrahydrofuran, dimethylformamide and methanol. In case of the epoxide **1** the membrane was treated with a solution of 3.5 ml hydrazine hydrate (80%) in 40 ml ethanol for 24 h and washed with methanol, acetic acid, 10% triethylamine in dimethylformamide, dimethylformamide and methanol yielded in the modified cellulose membrane **3**. In case of the epoxide **2** the membrane was treated with a solution of 20% piperidine in dimethylformamide for 15 min, washed with diethylformamide and methanol resulting in the modified membrane **3**.
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