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Polymer-bound Alkyltriazenes for Mild Racemization-free Esterification of Amino Acid and Peptide Derivatives

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Abstract: A novel tool for polymer-assisted solution phase (PASP) esterification of amino acid and peptide derivatives has been developed. When treated with carboxylic acids, polymer-bound alkyltriazenes react with a loss of nitrogen and transfer of the alkyl moiety to the carboxylate anion to form the corresponding alkyl esters. There are no limitations with regard to either the protecting groups or the nature of the amino acid. Furthermore no racemization occurs at the chiral centers of the amino acids as demonstrated by chiral GC-MS analyses. Alkyltriazene-resins were also applied successfully to the esterification of peptide acids and other peptidic structures, such as tripalmitoyl-*S*-glyceryl-cysteine (Pam_3Cys). The triazene-mediated esterification reaction is exceptionally mild, and there is no need for prior activation of the carboxy groups. This method is therefore particularly suitable for the alkylation of complex peptidomimetic structures prone to racemization and for acid-sensitive structures.

Keywords: polymer-assisted synthesis; polymer-bound reagents; carboxy protecting groups; alkylation; esterification; alkylaryltriazenes

INTRODUCTION

For decades in classical peptide chemistry and in multiple and combinatorial approaches [1,2] three of the most commonly used carboxy protecting groups have been the methyl-, allyl- and benzyl-groups [3]. The results are reported of the transfer of the three alkyl moieties to *N*-protected amino acid and peptide derivatives using polymer-bound alkyltriazenes (alkyltriazene-resin) prepared from the corresponding alkylamines.

Common alkylating reagents such as diazoalkanes, alkyl halides or dialkylsulfates are often toxic or moisture sensitive or even explosive [4]. Activation of the carboxy groups of peptides for esterification also shows significant disadvantages due to the long reaction times and racemization [5]. For this reason a novel esterification procedure has been developed for amino and peptide acids allowing the selective transfer of protecting groups (methyl, allyl, benzyl) as well as the introduction of additional functionality and structural diversity.

The new method is expected to be of special value in the field of complex peptidomimetics where common orthogonal protection is often at its limits, as well as for acid-sensitive structures. Moreover, the new and convenient derivatization tool fits well to our very early and recent developments in the trendy polymer assisted solution-phase (PASP) chemistry in our laboratory [6–9]. Last but not least PASP-syntheses combine the merits of classical solution-phase synthesis with the advantages of solid-phase synthesis, such as the use

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of reagents in high excesses, removal of the reagents by filtration and the possibility of automation [1,10].

Alkylaryltriazenes are activated under acidic conditions. They react with a loss of nitrogen and transfer of the alkyl moiety towards nucleophiles [11] (Scheme 1). As shown recently, polymer-bound alkyltriazene resins are particularly suitable for the esterification of carboxylic acids [12]. The reaction is best performed in dichloromethane or tetrahydrofuran containing up to 50 vol% methanol according to the solubility of the substrate.

The methylation reaction with the polymer reagent follows second order kinetics [12], corresponding to investigations that have been made earlier in solution [13]. The esterification reaction is free of isomerization with respect to the alkyl moiety which was shown for the reaction of *n*-butyltriazene-resin with benzilic acid using NMR experiments.

MATERIALS AND METHODS

Chemicals

Solvents, 4-nitrophenol, Cs_2CO_3 and $SnCl_2 \cdot 2H_2O$ were purchased from Fluka (Neu-Ulm, Germany), tert-butyl alkohol from Merck (Darmstadt, Germany) and tert-butyl nitrite from Aldrich (Steinheim, Germany).

Merrifield resin was purchased from Merck Biosciences (formerly: Novabiochem, Läufelfingen, Switzerland), Aldrich (Steinheim, Germany) and PepChem (Tübingen, Germany).

Tripalmitoyl-S-glyceryl-cysteine is available from EMC microcollections GmbH (Tübingen, Germany).

Chromatography and Spectroscopy

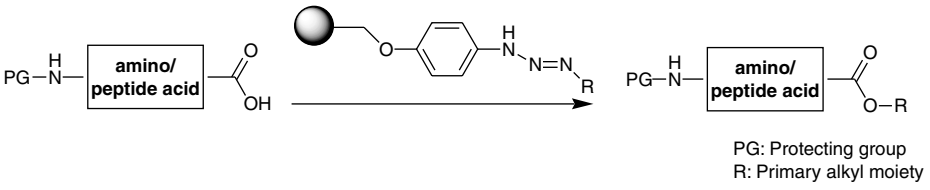
For HPLC analyses a Waters 600S System equipped with an autosampler unit (WISP 712) and UV detection at 214 nm was used. The HPLC column was a Nucleosil 100 C18, 250 × 2 mm, 5 µm particle size (Grom, Herrenberg, Germany). High resolution ES-FTICR-MS spectra were taken on a Daltonic APEX II spectrometer (Bruker, Bremen, Germany). 2D-NMR analyses were performed on a AMX 400 MHz spectrometer (Bruker Analytik, Bremen, Germany), with solutions in $CDCl_3$.

Chiral GC-EI-MS analyses for quantitative enantiomer analysis of the proteinogenic amino acids were done on a HP 6890/5973 (Hewlett Packard, Waldstetten, Germany). Fmoc deprotection of the samples was achieved by treatment with piperidine (10% in CH_2Cl_2) followed by side-chain deprotection using HCl/ethanol at 110 °C for 0.5 h (the side-chain carboxy groups of aspartic acid and glutamic acid are esterified). The final treatment with Tfa_2O/CH_2Cl_2 led to trifluoroacetylation of the *N*-termini and of unprotected side-chains. Histidine was methoxy carbonylated in the side-chain. The amino acid enantiomers were separated on the chiral GC stationary phase and detected by SIM-EI mass spectrometry. The chiral phase was either Lipodex-E [14] (30% in dimethylpolysiloxan) or Chirasil-Val [15].

Preparation of Triazene-Resins (General Procedure)

The preparation was performed according to the original protocol for smaller amounts of resin [12], which was scaled up to 10 g of resin.

Merrifield resin (10 g; initial loading 2.58 mmol/g; 25.8 mmol) and cesium carbonate (2 eq.; 51.6 mmol, 16.8 g) were suspended in DMF (90 ml) and 4-nitrophenol (2 M in DMF; 30 ml) was added. After stirring at 80 °C for 3 h the suspension was allowed to cool down to 60 °C. Acetic acid (50 ml) was added carefully and stirring was continued for another 0.5 h. The resin was washed thoroughly with DMF, CH_2Cl_2, MeOH/H_2O (4 : 1; v : v) and DMF. The resin was suspended in a 2 M solution of $SnCl_2 \cdot 2\,H_2O$ in DMF (120 ml), shaken at room temperature for 18 h, washed thoroughly with DMF, CH_2Cl_2 and MeOH/H_2O (4 : 1; v : v) and dried under reduced pressure.



Scheme 1 Smooth esterification of carboxy groups in amino acid and peptide derivatives using polymer-bound alkyl-triazenes.

The dried resin was suspended in CH_2Cl_2 (110 ml) and cooled to −18 °C. Cooled tert-butyl nitrite (10 eq.; 30.6 ml) was added dropwise under stirring within 30 min. After stirring at −18 °C for 1 h the reaction temperature was allowed to rise to room temperature during 3 h of additional stirring. The resin was washed thoroughly with CH_2Cl_2 and MeOH/H_2O (4 : 1; v : v) and dry THF.

The resin was suspended in a cooled (0 °C) solution of the appropriate amine (10 eq.; 258 mmol) in dry THF (100 ml), shaken for 0.5 h at 0 °C and 18 h at r.t. The resin was washed thoroughly with THF, CH_2Cl_2, MeOH/H_2O (4 : 1; v : v) and MeOH and dried under reduced pressure.

The final loading of the resulting alkyltriazene-resin was determined by elemental analysis for nitrogen, and ranged between 76% and >95% according to the initial loading of the Merrifield resin with chloride (as determined by elemental analysis for chlorine).

Alkylation of Amino Acid and Peptide Derivatives (General Procedure)

A solution of the amino acid or peptide derivative (1 mg) in CH_2Cl_2 containing up to 10% MeOH (1 ml) was added to the appropriate alkyltriazene-resin (10 eq.) and the resulting suspension was slightly stirred for 8 h at room temperature. The resin was then filtered off and washed twice with CH_2Cl_2 and MeOH. The combined filtrates were dried under reduced pressure and lyophilized from tert-butylalcohol/water (4 : 1, v : v). The reaction product was characterized using HPLC, ES-FTICR-MS and NMR [16,17]. As a representative example, the yield of methyl *N*-tert-butyloxycarbonyl-phenylalaninate was 83%. The NMR-data for methyl *N*-tert-butoxycarbonyl-phenylalaninate were: ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 1.33 (s, 9H), 3.00 (qd, 2H), 3.63 (s, 3H), 4.51 (qd, 2H), 4.89 (d, 1H), 7.03–7.23 (m, 5H); ^{13}C-NMR (400 MHz, $CDCl_3$) δ ppm: 28.68, 38.75, 52.61, 54.80, 80.32, 127.42, 128.94, 129.69, 136.39, 153.29, 172.76.

RESULTS AND DISCUSSION

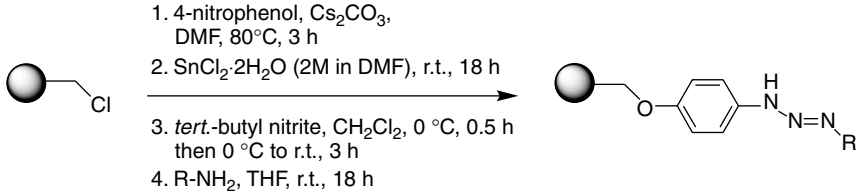
Preparation of Alkyltriazene-Resin

Alkyltriazene-resins were prepared in four steps on Merrifield resin (chloromethylated polystyrene resin crosslinked with 1% divinylbenzene). 4-Nitrophenol was attached to the resin by substitution of the chloride. The resin-bound 4-nitrophenol was reduced using tin dichloride dihydrate in dimethylformamide followed by diazotation with tert-butyl nitrite in dichloromethane. The diazonium chloride was reacted with different primary amines to yield a broad variety of alkyltriazene-resins (Scheme 2). The loadings of the polymer reagents ranged between 65% and 95% as determined by elemental analysis for nitrogen and they appeared to be storable at room temperature when kept dry and in the dark. In spite of a noticeable decrease in loading after long term storage (e.g. methyltriazene showed a loss of 47% after 30 months at 4 °C) no loss in product purities was observed.

Preliminary attempts to esterify *N*-terminally unprotected amino acids were not successful because of their poor solubility in the organic solvents necessary for triazene-resin mediated esterification reactions. A change towards more hydrophilic carrier materials could circumvent this limitation, which is the subject of ongoing investigations.

Optimization of Alkylation Conditions

In order to optimize the esterification reaction three different Fmoc protected amino acids (Fmoc-Phe-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Asp(OtBu)-OH) and three different alkyltriazene resins (methyl-, allyl- and benzyltriazene-resin) were used. The reaction proceeded best in dichloromethane containing up to 10 vol% of methanol. Optimized reaction conditions were 10 eq. of triazene and a reaction time of 8 h at room temperature (Table 1, entries 1, 2 and



Scheme 2 Alkyltriazene resins are prepared in four steps on Merrifield resin using primary alkylamines (R-NH_2) in the last step.

3). Longer reaction times using a lower excess of triazene led to incomplete conversions as well as to the formation of by-products. Products were lyophilized and analysed by HPLC, electrospray mass spectrometry (ES-MS) and NMR spectroscopy. Next a randomized collection of amino acids bearing a broad range of common protecting groups was esterified. This esterification procedure showed no incompatibility with any of the common protecting groups. Esterification of side chain unprotected amino acids such as Fmoc-Ser-OH and Fmoc-Tyr-OH was equally successful. Aspartic acid and glutamic acid derivatives could be esterified either *C*-terminally or in the side chain without rearrangement.

The highest product purities were obtained for methylation and ethylation reactions, whereas benzylation and allylation of Fmoc-Trp-OH, Fmoc-Met-OH and Fmoc-Cys(*t*Bu)-OH was somewhat less effective. The results of esterification reactions are shown in Table 1.

Table 1 Transfer of Carboxy Protecting Groups to Amino Acid Derivatives via Triazene Alkylation. Purities of the Alkylation Products have been Determined by HPLC ($\lambda = 214$ nm)

Entry	Amino acid derivative	HPLC-purity (%)		
		Methyl	Allyl	Benzyl
1	Fmoc-Phe-OH	>98	89	98
2	Fmoc-Arg(Pbf)-OH	91	85	85
3	Fmoc-Asp(OtBu)-OH	95	94	98
4	Fmoc-Gly-OH	>98	90	87
5	Fmoc-Ile-OH	93	96	98
6	Fmoc-Aib-OH	98	90	90
7	Fmoc-Chx-OH[a]	98	80	73
8	Boc-Phe-OH	>98	89	95
9	Fmoc-Ser-OH	95	80	72
10	Boc-Ser(Bzl)-OH	85	92	93
11	Boc-Tyr-OH	>98	79	80
12	Fmoc-Trp-OH	>98	64	68
13	Fmoc-Met-OH	97	66	51
14	Boc-Arg(Mts)-OH	98	88	87
15	Fmoc-Lys(Boc)-OH	>98	93	97
16	Fmoc-Lys(Mtt)-OH	94	93	95
17	Boc-Lys(Fmoc)-OH	98	97	98
18	Fmoc-Glu(OtBu)-OH	98	96	80
19	Fmoc-Asp(OH)-OtBu	>98	92	96
20	Fmoc-Glu(OH)-OtBu	97	84	88
21	Fmoc-Cys(Trt)-OH	98	70	61
22	Fmoc-Cys(tButhio)-OH	98	88	85

[a] Chx, 1-amino-1-carboxy-cyclohexane.

Variation of the Alkyl Moiety

In order to prove the generality of our concept Fmoc-Phe-OH was alkylated with a variety of alkyltriazene-resins. Amongst these were primary alkyl moieties as well as PEG-containing and even heterocyclic groups (Table 2).

In addition to reactions performed in separate vessels with single polymer-bound reagents, Fmoc-Phe-OH was treated with a small collection of five different alkyltriazene-resins in one pot yielding a mixture of five Fmoc-Phe esters. This mixture was analysed using the highly resolving micro-HPLC-ES-FTICR-mass spectrometry [16,17]. The UV chromatogram showed comparable conversion rates, whereas two compounds with basic groups showed much higher signal intensities in the contour plot of the HPLC-MS coupling. This was expected due to the higher protonation probability of these *N*-containing compounds (Figure 1).

However, attempts failed to react a mixture of amino acids with a mixture of alkyltriazene-resins. Two reasons seem to be responsible for this. On the one hand the reactivities of the alkyltriazene-resins differ as well as the p*K*a-values of the amino acid derivatives which leads to different reaction rates. Furthermore, the protonation probabilities of the reaction products are considerately different. This is reflected in the fact that some products were almost undetectable, whereas others gave very intense signals in MS analysis.

Esterification with Alkyltriazene-Resins is Racemization-Free

To investigate whether the alkylation performs under retention of configuration at the C_α atom all the Fmoc-protected proteinogenic amino acids were

Table 2 Esterification of Fmoc-Phe-OH using Different Alkyltriazene-resins

Entry	Alkyl moiety	HPLC-purity (%)
1	Methyl	>98
2	Ethyl	>98
3	*n*-Butyl	86
4	Benzyl	98
5	Allyl	89
6	13-Amino-4,7,10-trioxatridecyl	79
7	2-(Pyrid-2-yl)ethyl	80

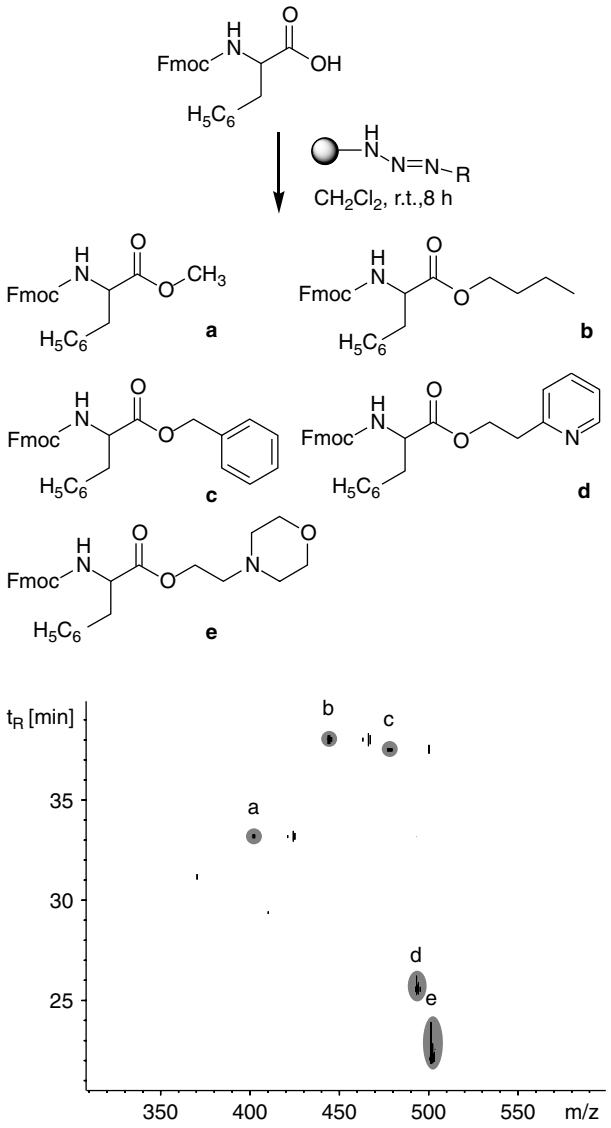


Figure 1 One pot alkylation of Fmoc-Phe-OH with a collection of five alkyltriazene-resins. The contour plot (m/z vs t_R) of HPLC-ES-FTICR-MS shows the five expected products. Compounds d and e show stronger signals due to the much higher protonation probability of these N-containing compounds.

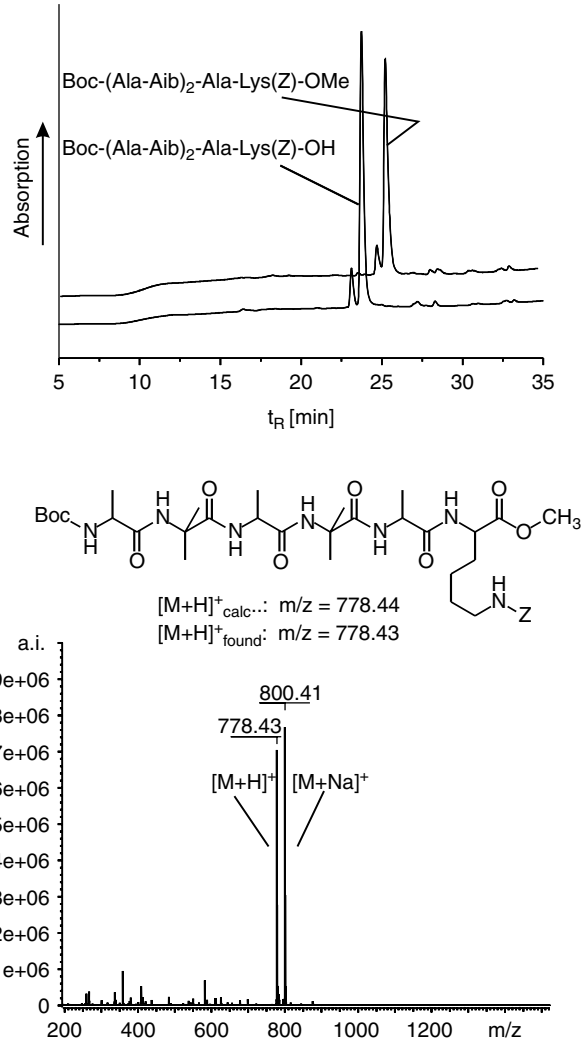


Figure 2 HPLC chromatogram of both educt and product of the methylation of the hexapeptide Boc-(Ala-Aib)$_2$-Ala-Lys(Z)-OH (above) and ES-FTICR-MS spectrum of the methylation product ($[M+H]^+_{calc.}$: 778.44; $[M+H]^+_{found}$: 778.43) (below).

ethylated using ethyltriazene resin. Treatment with piperidine/dichloromethane followed by hydrolysis with hydrochloric acid/ethanol and finally trifluoroacetylation of the *N*-termini with trifluoroacetic acid anhydride/dichloromethane led to volatile products suitable for analysis using chiral GC with SIM-EI-MS-detection [14,15]. No racemization was detected that could be attributed to the alkylation reaction. The higher value for cysteine was commonly observed due to the racemization tendency of cysteine derivatives under the analytical derivatization conditions.

Product purities were determined by HPLC measurements and found to be exceptionally good, except for Fmoc-His(Trt)-OEt and for Fmoc-Trp(Boc)-OEt. The results of these experiments are summarized in Table 3.

Alkylation of Peptide Acids

In the case of peptides a significant discrimination in the performance of triazene mediated alkylation was observed. Methylation showed good results, whereas for allylation and benzylation the reaction could

Table 3 Ethylation of the Carboxy Group of Fmoc Derivatives of All Proteinogenic Amino Acids Followed by Chiral GC-MS Analysis Showed no Significant Racemization and Exceptionally High Product Purities

Entry	Amino acid ester	HPLC (%)	D-Enantiomer[a] (%)	e.e. (%)	GC-column
1	Fmoc-Ala-OEt	98	0.61	98.78	a
2	Fmoc-Cys(Trt)-OEt	96	1.56	96.88	a
3	Fmoc-Asp(OtBu)-OEt	>98	0.26	99.48	b
4	Fmoc-Glu(OtBu)-OEt	>98	0.87	98.26	b
5	Fmoc-Phe-OEt	>98	0.14	99.72	b
6	Fmoc-Gly-OEt	>98	—	—	—
7	Fmoc-His(Trt)-OEt	85	0.04	99.92	b
8	Fmoc-Ile-OEt	>98	0.33	99.34	a
9	Fmoc-Lys(Boc)-OEt	96	0.27	99.46	a
10	Fmoc-Leu-OEt	98	0.39	99.22	a
11	Fmoc-Met-OEt	98	0.14	99.72	a
12	Fmoc-Asn(Trt)-OEt	95	0.53	98.94	a
13	Fmoc-Pro-OEt	97	0.41	99.18	a
14	Fmoc-Gln(Trt)-OEt	90	0.23	99.54	a
15	Fmoc-Arg(Pbf)-OEt	95	n.d.[b]	n.d	b
16	Fmoc-Ser(tBu)-OEt	>98	0.91	98.18	b
17	Fmoc-Thr(tBu)-OEt	98	0.39	99.22	a
18	Fmoc-Val-OEt	98	0.14	99.72	a
19	Fmoc-Trp(Boc)-OEt	77	0.27	99.46	b
20	Fmoc-Tyr(tBu)-OEt	98	0.38	99.24	a

a, Lipodex-E (30% in dimethylpolysiloxane); b, Chirasil-Val.
[a] Determined by comparison of peak areas of both enantiomers obtained by GC-MS analysis.
[b] Not determined.

not be driven to completion (Table 4). For example, methylation of Fmoc-Ile-Gly-OH was performed in 81% yield whereas benzylation of the same dipeptide acid gave a poor conversion of 52%. Interestingly neither a prolonged reaction time nor a higher excess of triazene resulted in a higher product purity, even though less starting material remained.

Next an *N*-protected hexapeptide acid which was readily dissolved in dichloromethane was alkylated. The HPLC chromatogram and the ES-FTICR-MS spectrum of the methylation product of the hexapeptide Boc-(Ala-Aib)$_2$-Ala-Lys(Z)-OH is shown in Figure 2. The conversion rate was about 100%. Neither by HPLC nor by MS was any remaining starting material detectable. The small peak on the left side of the main peak in the HPLC chromatogram belongs to a diastereomer in the starting material which resulted from partial racemization of L-alanine during a classical segment coupling step (not published).

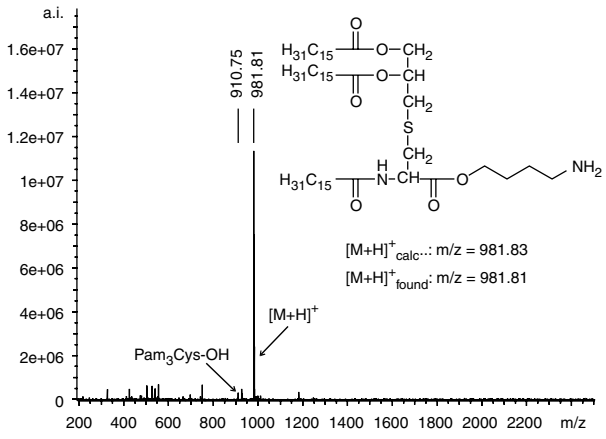


Figure 3 ES-FTICR-MS spectrum of crude 4-aminobutylated Pam$_3$Cys-OH ($[M+H]^+_{calc.}$: 981.83; $[M+H]^+_{found}$: 981.81).

Tripalmitoyl-*S*-glyceryl-cysteine (Pam$_3$Cys-OH), the synthetic *N*-terminus of lipoprotein from *Escherichia coli* has been long known for its

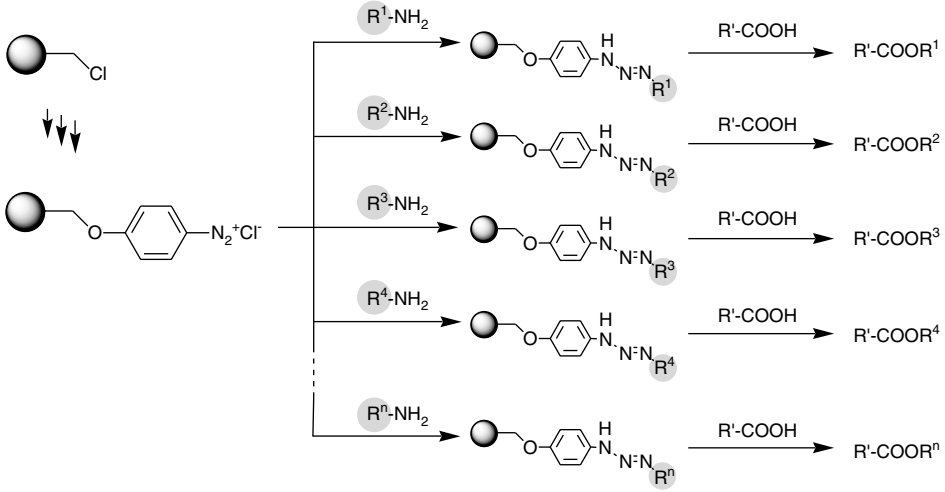
Table 4 Esterification of Simple Peptide Acids by Alkylating Polymer Reagents

Entry	Peptide acid	Alkyl moiety	$[M+H]^+$	HPLC (%)[a]
1	Fmoc-Ala-Ala-OH	Methyl	397	90
2	Fmoc-Ala-Ala-OH	Allyl	423	51 (47)
3	Fmoc-Ala-Ala-OH	Benzyl	473	52 (44)
4	Fmoc-Ile-Gly-OH	Methyl	425	81
5	Fmoc-Ile-Gly-OH	Allyl	451	54 (39)
6	Fmoc-Ile-Gly-OH	Benzyl	501	52 (39)
7	Fmoc-Gly-Gly-Gly-OH	Methyl	426	94
8	Fmoc-Gly-Gly-Gly-OH	Allyl	452	49(49)
9	Fmoc-Gly-Gly-Gly-OH	Benzyl	502	38(62)
10	Boc-Ala-Aib-OH	Methyl	289	82
11	Fmoc-Arg-Leu-OH	Methyl	510	62
12	Boc-Pro-Val-Aib-Aib-OH	Methyl	499	77
13	Boc-$(Ala\text{-}Aib)_2$-Ala-Lys(Z)-OH	Methyl	778	84
14	Boc-$(Aib\text{-}Ala)_5$-OH	Methyl	899	48

[a] Purities (peak area, 214 nm); values in parentheses indicate remaining starting material.

Table 5 Results of the Alkylation of the Lipoamino Acid Tripalmitoyl-S-glyceryl-cysteine (Pam_3Cys-OH) using Triazene Polymer Reagents Derived from Methylamine, 1,4-Diaminobutane and 1,13-Diamino-4,7,10-trioxatridecane

Entry	Alkyl moiety	Molecular formula	$[M+H]^+$ calc.	$[M+H]^+$ found
1	Methyl	$C_{55}H_{106}NO_7S$	924,769	924,768
2	4-Aminobutyl	$C_{58}H_{113}N_2O_7S$	981,83	981,83
3	13-Amino-4,7,10-trioxa-tridecyl	$C_{64}H_{125}N_2O_{10}S$	1113,905	1113,911



Scheme 3 Alkyltriazene-resins are highly versatile polymer-bound reagents for parallel production of collections of amino acid and peptide derivatives. Examples: R^1, Me; R^2, *n*Bu; R^3, Bzl; R^4, 2-(pyrid-2-yl)ethyl; R^5, 2-(morpholin-1-yl)ethyl.

immunological adjuvant activity in vaccine constructs [18–20] and was recently found to act as a ligand for toll-like receptor-2 (TLR2) [21,22]. *C*-terminal modification using 4-aminobutyltriazene-resin and 13-amino-4,7,10-trioxa-tridecyltriazene-resin led to more polarity of the lipid and thus made the alkylation products soluble in a broader range of polar solvents. This example also demonstrates the introduction of an *N*-terminally free aminoalkyl ester group in one step to a structurally non-trivial compound. Alkylation results are shown in Table 5. Figure 3 shows the ES-FTICR-mass spectrum of the aminobutylated lipoamino acid.

In summary, a novel tool has been introduced for the smooth and efficient PASP esterification of amino acid and peptide derivatives. The above mentioned substrates were esterified using only one kind of reagent that can be equipped with the desired alkyl moiety to be transferred (Scheme 3). Also, there was no need to re-optimize reaction conditions for each and every esterification reaction. Thus alkyltriazene-resins are highly versatile polymer-bound reagents which have high potential in preparative and analytical peptide and peptidomimetic chemistry.

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