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Acid-Stable Ester Linkers for the Solid-Phase Synthesis of Immobilized Peptides

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A series of N-terminally Fmoc-protected linkers of the general formula Fmoc-X–CO–O–Y–COOH have been prepared, where X is –NH–CH_2–CH_2- or -*p*-(aminomethyl)phenyl- and Y is –$(CH_2)_n$– (n is 1 or 4) or -*p*-(methyl)phenyl-. These linkers can easily be covalently attached via their C-terminal carboxyl group to a resin bearing a free amino group. After cleavage of the N-terminal Fmoc group, the linkers can be extended by standard solid-phase peptide synthesis techniques. These ester linkers are acid-stable and resistant to the base-mediated diketopiperazine formation that often occurs during the synthesis of ester-bound peptides; they are stable at neutral pH in aqueous buffers for days but can be effectively cleaved with 0.1 m NaOH or aq. ammonia within minutes or hours, respectively. These properties make these ester handles well suited for use as linkers for the solid-phase peptide synthesis of immobilized peptides when the stable on-resin immobilization of the peptides and the testing of their biological properties in aqueous buffers at neutral pH are necessary.

Introduction

Compounds immobilized on solid supports are broadly applicable, e.g., in the affinity purification of proteins,[1] the identification of new protein targets for immobilized ligands[2] or in combinatorial approaches for the identification of new protein ligands and drug leads.[3]

Peptides are frequently used as ligands for targeting proteins of pharmacological interest. Solid-phase peptide synthesis is a common method for the generation of new ligands in the form of resin-bound combinatorial libraries.[4] The most frequently employed variant of solid-phase peptide synthesis uses N^{α}-Fmoc and side-chain tBu-protected amino acids[5] and involves elongation of a peptide sequence from the C- to the N-terminus and a final acid-mediated cleavage of protecting groups of the amino acid side-chains together with the simultaneous cleavage of the linker moiety between the peptide C-terminus and the solid support (resin). On the other hand, stable peptide immobilization on a solid-phase support after completion of a synthesis, i.e., after cleavage of the acid-labile side chain protecting groups, requires that the linkage between the peptide and the resin is resistant to concentrated trifluoroacetic acid (TFA), which is usually used for deprotection. This can be achieved simply by coupling the first C-terminal amino acid to the resin-bound primary amino group, forming a TFA-stable amide bond. However, this approach does not allow nondestructive cleavage of the final peptide from the resin. A nondestructive cleavage/release of compounds from the solid support can be useful in certain situations, such as monitoring the quality of the synthesis of immobilized peptides or especially in combinatorial approaches for identifying the peptides on "positive" resin beads by mass spectrometry after biological screening libraries of compounds immobilized on beads.[3a,c–g]

A variety of linkers and cleavage strategies have been developed for solid-phase peptide chemistry.[6] Some of these strategies, such as photochemical approaches,[7] metal-assisted strategies[8] or so-called "safety catch linkers",[9] offer alternatives to acid-mediated cleavage of peptides from solid supports. However, these approaches often involve the use of less-straightforward chemistries or reagents that can cause side reactions (Ref.[6b] and references therein). In this respect, connecting the peptide carboxy terminus to the resin-bound 4-hydroxymethyl benzoic acid (HMBA) linker via an ester bond seems to be the simplest and best solution.[10] However, esterification of the hydroxyl group with an N^{α}-Fmoc-protected amino acid can be accompanied by incomplete couplings[11] or side reactions such as base-mediated Fmoc cleavage[12] and racemization.[13] Importantly, resin-bound C-terminal amino acid esters are also prone to diketopiperazine formation during peptide synthesis.[5,14] Diketopiperazine formation and subsequent cleavage of the peptides from the resin can be a serious complication in combinatorial synthesis of peptide mixtures where an equimolar distribution of peptides is expected.

The aim of this work was to develop a linker that (i) can be easily attached to a resin bearing free amino groups, (ii) is stable during piperidine-mediated Fmoc deprotection, (iii) is resistant to the racemization and diketopiperazine formation often accompanying peptide synthesis, (iv) is resistant to the TFA used for amino acid side chains deprotection, (v) is stable in aqueous buffers of neutral pH, which are common media for many biological assays and, finally, (vi) can be cleaved under conditions that will not damage the synthesized peptide and will allow mass spectrometric characterization. Such a linker should have properties (summarized in Figure 1) suitable for

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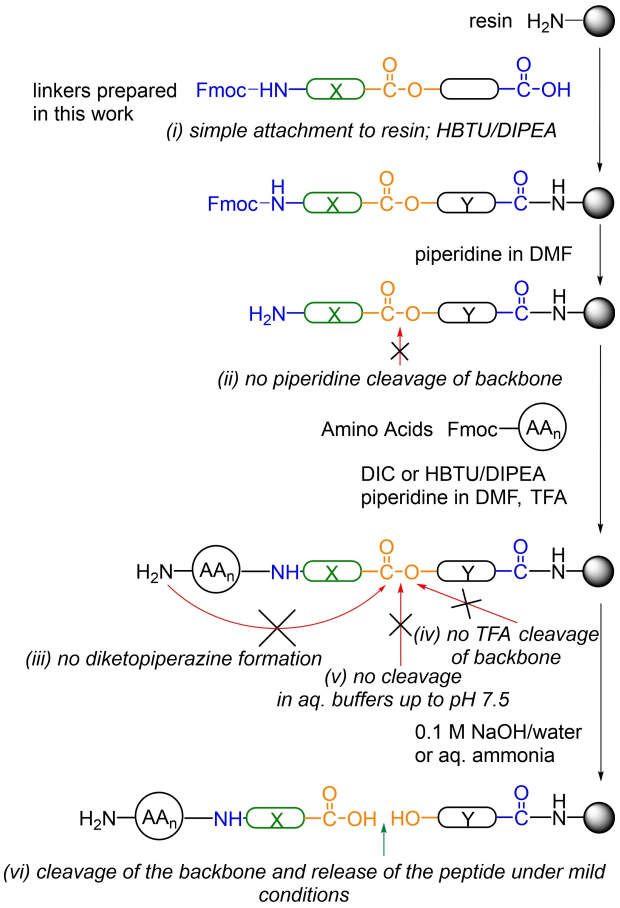
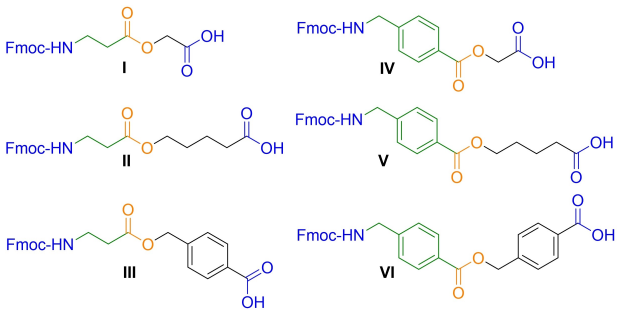


Figure 1. General principles and results of this work. The blue C-terminal carboxyl group enables a simple and stable attachment to the amino-substituted resin, and cleavage of the blue Fmoc-protected amino group enable elongation with amino acids by classic Fmoc/*t*-Bu peptide synthesis techniques. The chemical structure of the green X part of the linkers provides resistance to diketopiperazine formation during peptide synthesis, the black Y part of the linker modulates the stability at neutral pH and, finally, the central orange ester bond enables the cleavage of the peptide from the resin under mild alkaline conditions.

both the synthesis and screening of resin-bound combinatorial libraries of compounds and could be an alternative to linkers cleavable with UV light,[3d,7] cleavable with acids stronger than TFA (e.g. PAM ester linker[3e]) or methionine-containing linkers[3f,g] cleavable with CNBr, and could help to avoid side reactions connected with these reactions and reagents.

In 1994, Bray et al.[10c] published a series of ester handles of general formulas Fmoc-AA$_x$–O-*p*-(methyl)phenyl-COOH or Fmoc-AA$_x$–O–CH$_2$-COOH (where AA$_x$ is an α-amino acid) and explored their properties in conjunction with multipin peptide synthesis. In 1991, Valerio et al.[15] prepared an ester linker, Fmoc-NH–CH$_2$–CH$_2$–CO–O-*p*-(methyl)phenyl-COOH, bearing a β-alanine at the N-terminus that provides stability to diketopiperazine formation after cleavage of the Fmoc group from the linker. Inspired by these studies, we prepared and characterized a series of six ester handles, **I–VI**, containing N^{α}-Fmoc-protected β-alanine or *p*-(aminomethyl)benzoic acid moieties (Scheme 1), and the presence of these groups at the N-terminus of the linkers prohibits the formation of undesirable diketopiperazine



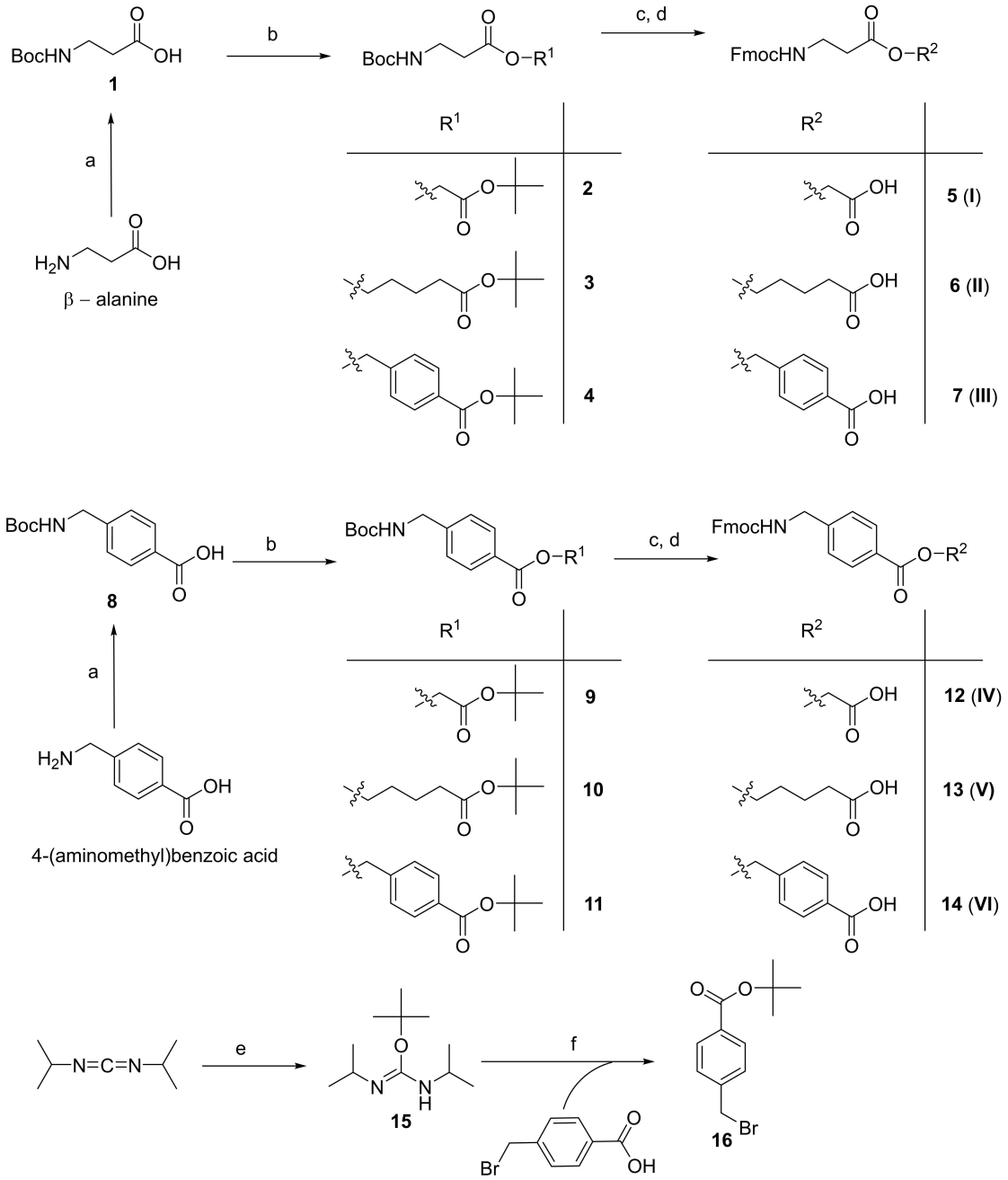
Scheme 1. Linkers for the solid-phase peptide synthesis prepared in this work. The color scheme is the same as in Figure 1. Linker **III** has already been prepared by Valerio et al.[15]

byproducts during the peptide synthesis. Linker **III** is identical to the Fmoc-NH–CH$_2$–CH$_2$–CO–O-*p*-(methyl)phenyl-COOH linker prepared by Valerio et al.[15] We prepared a series of model peptides on these linkers, and we investigated in detail their stabilities in aqueous buffers of different pH values, which is a crucial property for biological applications of immobilized peptides. We found that the ester linkers prepared here can be useful as acid-stable and base-labile linkers for the solid-phase synthesis of resin-immobilized peptides and their applications.

Results and Discussion

Solution-phase synthesis of compounds

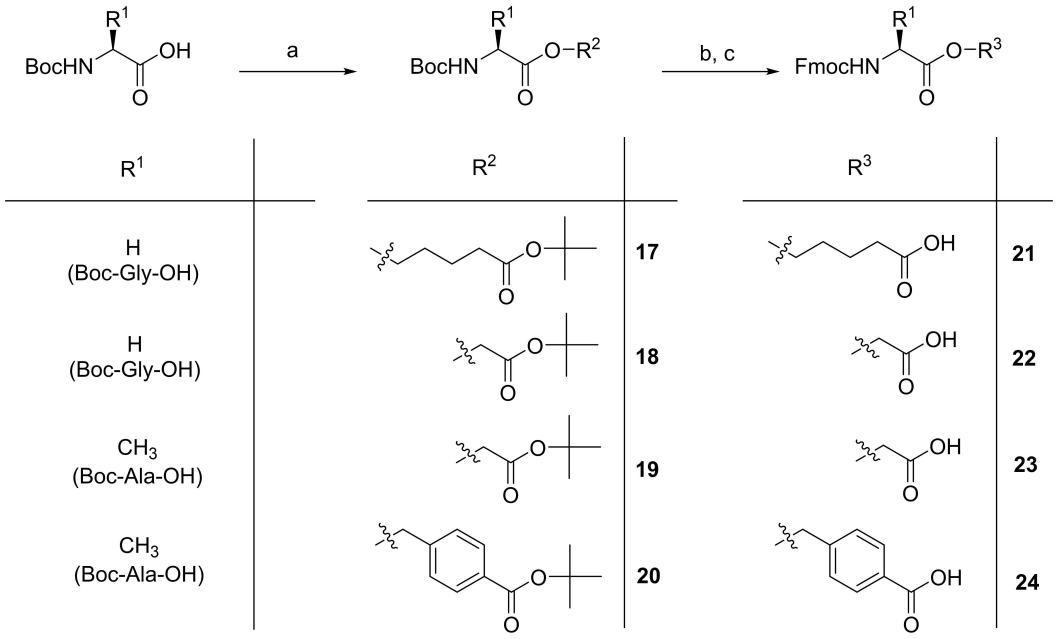
Scheme 2 shows our strategy for the synthesis of target compounds **5** (**I**)–**7** (**III**) and **12** (**IV**)–**14** (**VI**), namely, a straightforward three-step synthetic sequence starting from Boc-protected acids **1** and **8**. Recently, we directly alkylated propargylamine with *tert*-butyl 5-bromovalerate in acetonitrile in the presence of potash.[16] Here, we applied these reaction conditions for the esterification of **1** or **8** with the corresponding brominated derivatives. Using this reaction, we prepared a series of intermediates, **2**–**4** and **9**–**11**, in high yields (approximately 90%). Another positive feature of this straightforward protocol is the fact that after filtering off the solid potassium carbonate from the reaction mixture, the filtrate is concentrated under reduced pressure, and the oily residue is loaded directly on a column without any further aqueous work-up. The starting *tert*-butyl 2-bromoacetate is commercially available, and *tert*-butyl 5-bromovalerate was prepared by a previously published method.[16] In the case of the synthesis of *tert*-butyl 4-(bromomethyl)benzoate,[16] we tested different esterification methods using a series of condensation agents, such as DIC and PyBroP, but we ultimately found that the highest yield was obtained by heating isourea **15** with 4-(bromomethyl)benzoic acid in DCM (Scheme 2, f). In the next step, both the Boc and *t*Bu acid-labile groups of intermediates **2**–**4** and **9**–**11** were cleaved by treatment with TFA in DCM with rapid liberation of isobutene and carbon dioxide. The fully deprotected intermediates were not isolated but dissolved in aqueous sodium hydrogen carbonate solution, and then the free amino group



Scheme 2. Reagents, conditions and yields: (a) Boc_2O, Na_2CO_3, water and dioxane, 1 h at 0 °C then at rt overnight (87% for **1**, 60% for **8**); (b) *tert*-butyl 2-bromoacetate for **2** and **9**, *tert*-butyl 5-bromovalerate for **3** and **10**, **16** for **4** and **11**, K_2CO_3, AcCN, 60 °C overnight (92% for **2**, 88% for **3**, 87% for **4**, 92% for **9**, 87% for **10**, 91% for **11**); (c) TFA, DCM, 1 h at 0 °C then 2 h at rt; (d) Fmoc-Osu, $NaHCO_3$ water, dioxane, 1 h at 0 °C then at rt overnight (95% for **5**, 94% for **6**, 92% for **7**, 94% for **12**, 92% for **13**, 90% for **14**, each yield is over two steps); (e) *tert*-butanol, CuCl, 6 d at rt (88%), (f) **15**, DCM, at 40 °C for 24 h (88%).

was acylated with Fmoc-Osu. After classical aqueous work-up and trituration of the solids, pure products **5** (**I**)–**7** (**III**) and **12** (**IV**)–**14** (**VI**) were isolated in excellent yields (above 90% for two steps).

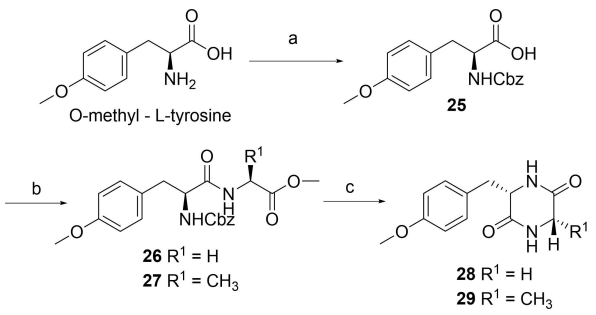
As mentioned in the Introduction, dipeptides attached by ester bonds to HMBA immobilized on resin can undergo intramolecular aminolysis.[17] This process is accelerated by piperidine, which is used for cleavage of the N^{α}-Fmoc protecting group and leads to diketopiperazine side-products. The structures of linkers **5** (**I**)–**7** (**III**), derived from β-alanine, and linkers **12** (**IV**)–**14** (**VI**), derived from 4-(aminomethyl)benzoic acid, preclude the formation of diketopiperazines, as they do not allow the creation of a stable six-membered ring after attachment of an additional α-amino acid and cleavage of its Fmoc group. In this respect, we sought to determine if ester linkers having a standard α-amino acid moiety at their N-terminal part, e.g., the handles prepared by Bray et al.[10c] (Fmoc-AA_x–O-*p*-(methyl)phenyl-COOH or Fmoc-AA_x–O–CH_2-COOH), which are mentioned in the Introduction, are prone or resistant to diketopiperazine formation after cleavage of their



Scheme 3. Reagents, conditions and yields: (a) *tert*-butyl 5-bromo valerate (for **17**), *tert*-butyl 2-bromo acetate (for **18** or **19**) or **16** (for **20**), K_2CO_3, AcCN, at 60 °C overnight (36% for **17**, 91% for **18**, 86% for **19**, 71% for **20**); (b) TFA, DCM, at 0 °C for 1 h then for 2 h at rt; (c) Fmoc-Osu, $NaHCO_3$ water, dioxane, for 1 h at 0 °C then at rt overnight (91% for **21**, 83% for **22**, 89% for **23**, 90% for **24**).

N-terminal Fmoc group (see Investigation of diketopiperazine formation below). Therefore, linkers **21**–**24** derived from glycine and alanine were prepared (Scheme 3). The synthetic strategy was the same as that used for compounds **5** (**I**)–**7** (**III**) and **12** (**IV**)–**14** (**VI**). Boc-Gly and Boc-Ala were reacted with protected bromo esters to give corresponding intermediates **17**–**20**. Surprisingly, unlike the other intermediates, product **17** was isolated in a lower yield (36%), and no modifications of the reactions conditions lead to better results. The last two synthetic steps, acid deprotection and a reaction with Fmoc-Osu, proceeded smoothly with high cumulative yields (83–91%) for compounds **21**–**24**.

Next, we prepared model cyclic compounds **28** and **29** (Scheme 4). They were designed to represent "standards" for the easy detection of potential diketopiperazine products in reaction mixtures after Fmoc deprotection of the amino acids attached to resin-bound linkers **21**–**24**. Thus, the free amino group of O-methyl-L-tyrosine was Cbz protected. Then, the PyBroP-mediated reaction of intermediate **25** with Gly-OMe·HCl or Ala-OMe·HCl led to dipeptides **26** or **27**, respectively. Catalytic hydrogenolysis of **26** or **27** afforded the free amino dipeptides, which quickly underwent spontaneous cyclization and afforded products **28** or **29**, respectively.



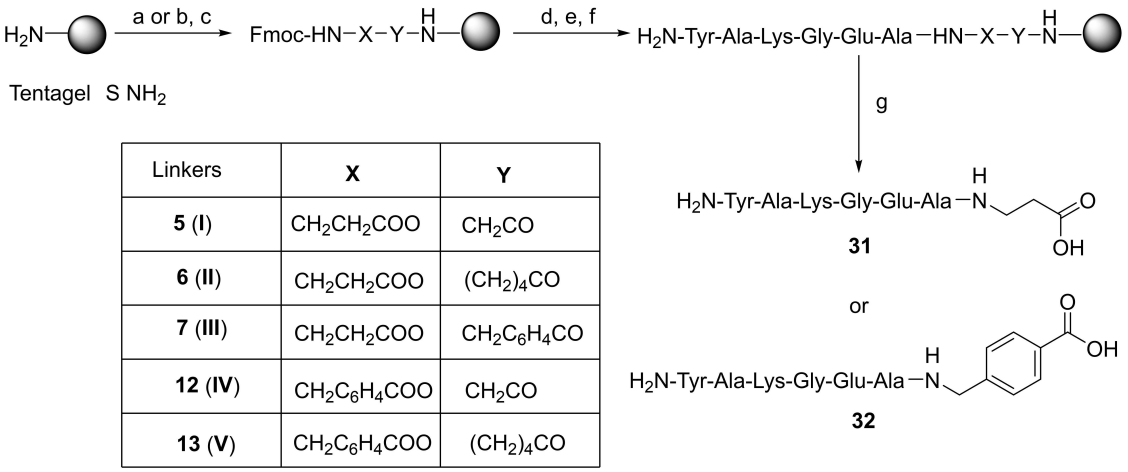
Scheme 4. Reagents, conditions and yields: (a) Cbz-Cl, Na_2CO_3, water and dioxane, at 0 °C for 1 h then at rt overnight (77%); (b) Gly-OMe·HCl or Ala-OMe·HCl, PyBroP, DIPEA, DMF, overnight at rt (70% for **26**, 69% for **27**); (c) 10% Pd/C, H_2, 15 psi, methanol at rt (63% for **28**, 65% for **29**).

The purities of compounds **5** (**I**)–**7** (**III**), **12** (**IV**)–**14** (**VI**), **21**–**24**, and **28**–**30** (Fmoc-piperidine adducts) were determined by RP-HPLC. The chromatograms are shown in Figures S1–S13 (see the Supporting Information).

Solid-phase peptide synthesis

For the synthesis of model peptide sequences, we chose a standard Fmoc strategy that is fully compatible with automated synthesis in peptide synthesizers and can be used to test the stability of the linker ester bonds in the presence of piperidine and TFA. Linker **14** (**VI**) could not be tested due to its negligible solubility in DMF and other commonly used solvents, and thus it was excluded from further experiments.

A schematic of the synthesis of model peptides **31** and **32** is shown in Scheme 5. Briefly, DMF-swollen beads of resin bearing free amino groups in plastic syringes equipped with Teflon frits were treated with linkers **5** (**I**)–**7** (**III**) or **12** (**IV**) and **13** (**V**) using either HBTU/DIPEA twice per hour or PyBroP/DIPEA for **8** and **17** h as condensation agents. Any remaining free amino groups were capped with acetic anhydride, and the Fmoc groups were cleaved, which enabled spectrophotometric quantitative estimation of the linker loading on the resin. Then, syringes with the resins were installed in an automatic peptide synthesizer, and a Tyr-Ala-Lys-Gly-Glu-Ala peptide chain was built using a



Linkers	X	Y
5 (**I**)	CH_2CH_2COO	CH_2CO
6 (**II**)	CH_2CH_2COO	$(CH_2)_4CO$
7 (**III**)	CH_2CH_2COO	$CH_2C_6H_4CO$
12 (**IV**)	$CH_2C_6H_4COO$	CH_2CO
13 (**V**)	$CH_2C_6H_4COO$	$(CH_2)_4CO$

Scheme 5. Reagents and conditions: (a) HBTU, DIPEA, **5**–**7** or **12**–**13**, DMF, 2× for 1 h; (b) PyBroP, DIPEA, **5**–**7** or **12** and **13**, DMF, for **18** and 7 h; (c) Ac_2O, DIPEA, DMF, 2× for 15 min; (d) 20% piperidine/DMF for 5 and 20 min; (e) Fmoc solid-phase peptide synthesis automated cycles with individual amino acids (see Methods); (f) 5% TIPS/TFA 2× for 1 h; (g) 100 mM NaOH, 45 min for **5**–**7**, 24 h for **12** and **13**. NH_2 denotes the N-terminus of the peptides.

standard Fmoc solid-phase peptide synthesis protocol. Acid-labile protecting groups (Boc and *t*Bu) on the side chains of Tyr, Lys and Glu were removed by treatment with an acidic cocktail. The cleavage solutions were filtered off from the resin, and the filtrates were concentrated and analyzed by HPLC. No acid-catalyzed linker hydrolysis products were found. Finally, the linker ester bond was saponified with an aqueous solution of 0.1 M NaOH. The linkers derived from β-alanine were cleaved in 45 min, and the linkers **12** (**V**) and **13** (**VI**) derived from 4-(aminomethyl)benzoic acid were cleaved in 24 h because we expected the carboxyphenyl ester moieties to be more stable to hydrolysis. Later (see below, *Stability of linkers*), the hydrolytic stabilities of the linkers were investigated, and the results are discussed below in detail. Briefly, these experiments confirmed that a longer cleavage time was suitable for **13** (**V**), but for **12** (**V**), 45 min in 0.1 M NaOH would be generally sufficient.

The loadings on the linkers and the yields after cleavage of the peptides from the resin are summarized in Table 1. Similar loadings of linkers **5** (**I**)–**7** (**III**) and **12** (**IV**) and **13** (**V**) were achieved with both condensation agents. It seems that the loadings of linkers **12** (**IV**) and **13** (**V**) were generally slightly lower than the loading of linkers **5** (**I**)–**7** (**III**), probably due to a lower reactivity of the benzene-bound carboxylic group.

RP-HPLC analyses (Figures S14–S19 in the Supporting Information) of crude peptides **31** (entries 1–6) revealed high purities for the products synthesized with linkers **5** (**I**)–**7** (**III**); all over 90%. In contrast, crude peptides **32** (entries 7–10), synthesized on linkers **12** (**IV**) or **13** (**V**) (Figures S20–S23 in the Supporting Information), were contaminated with several impurities, and their purities were approximately 75%, which is still satisfactory. These lower yields could be explained by the longer treatment with aqueous sodium hydroxide, which can damage peptides. Shorter cleavage times would likely provide purities similar to those of linkers **5** (**I**)–**7** (**III**). The RP-HPLC profiles of HPLC-purified peptides **31** and **32** (Entries 1–10) are shown in Figures S24–S33 (see the Supporting Information).

The alkaline cleavage of the peptides synthesized on resin-bound linkers **I–V** resulted in compounds with C-terminal β-alanine (e.g., **31** in Scheme 5) or C-terminal 4-(aminomethyl) benzoic acid groups (e.g., **32** in Scheme 5). This is consistent with the purpose of linkers **I–V**, which are designed for the stable immobilization of peptides for affinity purification of

Table 1. Summary of the syntheses of compounds **31** and **32** on TentaGel resin using linkers **I–V**.

Compounds	Entry/Linker	Loading [%][c]	Purity [%][d]	Yield [%][e]	HRMS[f]
31	Entry (1)/**5** (**I**)[a]	83	91	64	709.3527/731.3323
	Entry (2)/**6** (**II**) [a]	91	94	60	709.3528/731.3308
	Entry (3)/**7** (**III**)[a]	91	98	67	709.3525/731.3324
31	Entry (4)/**5** (**I**)[b]	87	93	61	709.3502/731.3314
	Entry (5)/**6** (**II**)[b]	93	93	64	709.3531/731.3307
	Entry (6)/**7** (**III**)[b]	93	97	69	709.3539/731.3311
32	Entry (7)/**12** (**IV**)[a]	84	73	54	771.3664
	Entry (8)/**13** (**V**)[a]	81	77	59	771.3693/793.3454
32	Entry (9)/**12** (**IV**)[b]	87	75	55	771.3667
	Entry (10)/**13** (**V**)[b]	86	75	51	771.3662

[a] Attachment of the linker to the resin with HBTU and DIPEA or [b] PyBroP and DIPEA. [c] Yield (relative to the amount of unsubstituted resin) of the loading of the linkers on the resin determined by spectrophotometric measurement of the adduct of dibenzofulvene and piperidine **30**. [d] Relative purities of the crude peptides determined by integration of their HPLC chromatograms. [e] Final yields of HPLC-purified peptides relative to the initial loading of resin. [f] HRMS-MALDI, $[M+H]^+/[M+Na]^+$.

proteins or similar biological applications and not primarily for the synthesis of peptides with a native C-terminus. It also means that the linkers can serve as integral parts of "spacers" separating the target ligand (e.g., a peptide) from the resin. Separating immobilized ligands from the solid support by spacers is a common strategy that can enhance the probability of affinity capture, especially for larger proteins.

Investigation of diketopiperazine formation

As previously mentioned (see Solution synthesis of compounds), dipeptides with an ester bond at the C-terminus can undergo spontaneous cyclization to form 6-membered rings under basic conditions. We expected that such side reactions would occur in the case of linkers **21**–**24**, which are derived from glycine and alanine. Hence, we performed model experiments and tested whether the formation of diketopiperazines **28** and **29** can occur after piperidine deprotection of the first Fmoc-amino acid attached to the linker. The mechanism for diketopiperazine formation is depicted in Scheme 6. Thus, **21**–**24** were attached to the resin via their carboxylic groups, and this was followed by the capping of the remaining free amino groups on the resin, Fmoc group removal with piperidine, and elongation of the immobilized linker with FmocTyr(OMe). Finally, the resins were treated with piperidine, and cleavage mixtures were subjected to the work-up described in detail in the Experimental section.

Figure 2 shows the HPLC chromatogram of a piperidine/DMF solution that was used for deprotection of the Fmoc group from Fmoc-Tyr(OMe) bound to linker **24** attached to the resin. This chromatogram was superimposed on HPLC chromatograms of model diketopiperazine **29** and fluorenyl-piperidine adduct **30**. The identical retention times (8.8 min) of diketopiperazine **29** and a compound formed in the piperidine solution after the reaction with Fmoc-Tyr(OMe)-linker **24** attached to the resin indicate the formation of the expected byproduct. Similar results were also obtained for linkers **21**–**23**, and their chromatograms are shown in Figures S34–S36, respectively (see the Supporting Information).

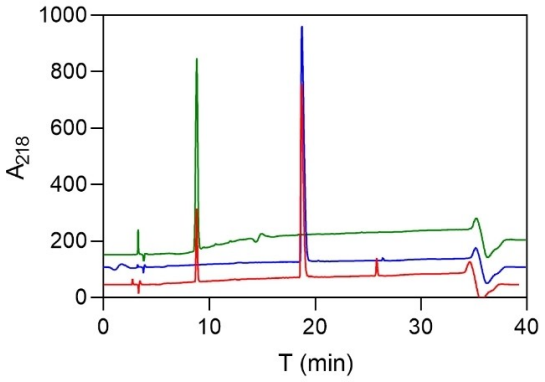
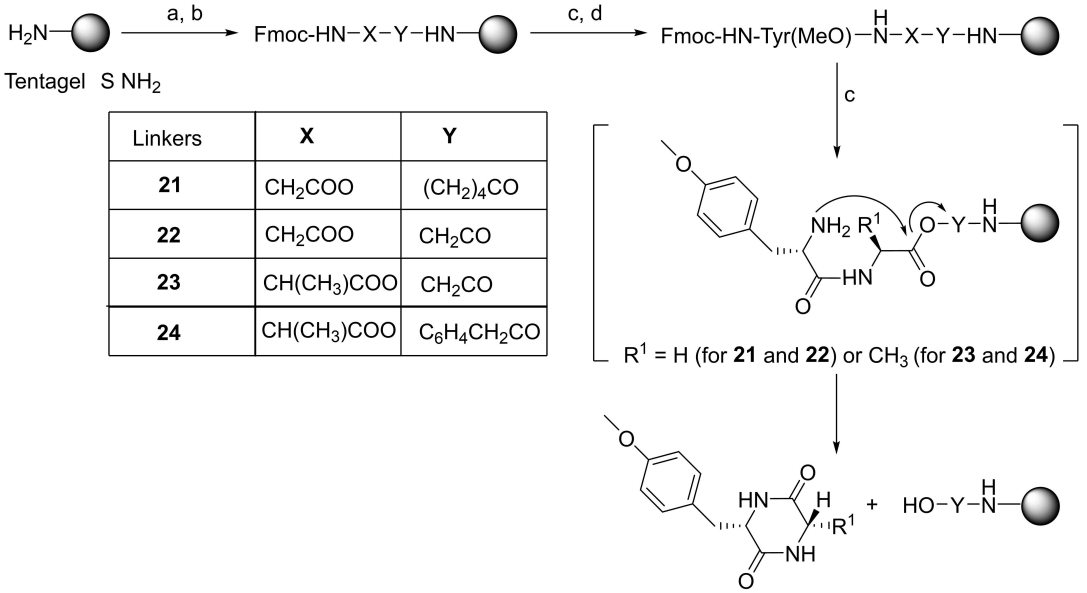


Figure 2. Superposition of the HPLC profiles (measured using Method 1, see Experimental) of piperidine solutions collected after treatment with resin-bound Fmoc-Tyr(OMe)-linker **24** (lower profile in red), 1-(9H-fluoren-9-ylmethyl)piperidine **30** (middle profile in blue) and previously prepared model diketopiperazine **29** (upper profile in green).

Compounds with HPLC retention times identical to those of model diketopiperazines **28** and **29** were isolated, and their structures were confirmed by spectroscopic methods. In the case of linker **21**, the amount of **28** isolated was low, albeit detectable, but the yields of diketopiperazine byproducts formed from linkers **22**, **23** and **24** were 43%, 54% and 44%, respectively.

Finally, by analogy with the experiments described in Scheme 6, we also attached Fmoc-Tyr(OMe) to linkers **I–V**, and



Linkers	X	Y
21	CH_2COO	$(CH_2)_4CO$
22	CH_2COO	CH_2CO
23	$CH(CH_3)COO$	CH_2CO
24	$CH(CH_3)COO$	$C_6H_4CH_2CO$

Scheme 6. Diketopiperazine formation on resin-bound linkers **21**–**24**. Reagents and conditions: (a) PyBroP, DIPEA, **21**–**24**, DMF, for 18 and 7 h; (b) Ac_2O, DIPEA, DMF, 2×15 min; (c) 20% piperidine/DMF 5 and 20 min; (d) Fmoc-Tyr(OMe)-OH, DIC, DMF, 2×1.5 h.

piperidine solution after the cleavage of the Fmoc group was analyzed. Chromatograms shown in Figure S37 did not reveal any traces of hypothetical diketopiperazine products. The results clearly show that the designed linkers bearing β-alanine (**5** (**I**)–**7** (**III**)) or 4-(aminomethyl)benzoic acid (**12** (**IV**) and **13** (**V**)) species in their structures prohibit the formation of diketopiperazines.

Stability of linkers

Linkers **5** (**I**), **6** (**II**), **7** (**III**), **12** (**IV**) or **13** (**V**) were attached to TentaGel® S NH_2 Resin, and the model peptide, Tyr-Ala-Lys-Gly-Glu-Ala, was synthesized on each of the linkers. The stability of each of the ester bonds in the linkers was investigated in aqueous solutions with different pH values: 50 mM citrate/NaOH at pH 6.1; 50 mM potassium phosphate at pH 6.5, 7.0, 7.5 and 8.0; 200 mM sodium bicarbonate/sodium carbonate at pH 8.7; and 50 mM glycine/NaOH at pH 10.5. The resin was incubated in each of these buffers, and the release of the cleaved products (peptide **31** for linkers **5**–**7** or peptide **32** for linkers **12** and **13**) into the supernatant was monitored by HPLC (see Methods). The results were compared to the effects of aqueous 100 mM NaOH under the same conditions (Figure 3). According to the results shown in Figures 3A-3E, the pH stability of linkers **5** (**I**), **6** (**II**), **7** (**III**), **12** (**IV**) and **13** (**V**) in aqueous buffers can be ranked approximately as follows (from the most stable to the least stable): **13** (**V**) > **12** (**IV**) ~**7** (**III**) > **6** (**II**) > 5 (**I**). This stability order can be explained by considering the chemical reactivities of the respective compounds. Alkyl benzoate **13** (**V**) is the most stable under aqueous basic conditions due to the electron donating effect of its benzene ring. The stability of **12**(**IV**) is ranked second because its sensitivity to nucleophilic attack is increased more by the "leaving group ability" of the methyl carboxamide group. Linker **7** (**III**), which has a stability similar to that of **12**, is composed of more reactive alkanoic acid, but its effect is counterbalanced by the electron-donating 4-(carboxamide)phenylmethyl group. Alkyl alkanoate **6** (**II**) showed the penultimate reactivity, and finally, the most reactive linker is **5** (**I**) because this compound, in addition to being an alkanoic acid, also contains an "easily leaving" methyl carboxamide group.

Linker **13** (**V**) is completely stable up to pH 8.0; only approximately 10% of the peptide was released at pH 10.5 after 48 h and even 0.1 M NaOH did not quantitatively cleave the peptide from the resin during the first 30 min (Figure 3E). Linkers **7** (**III**) (Figure 3C) and **12** (**IV**) (Figure 3D) share similar pH stabilities and show negligible cleavage up to pH 8 and approximately 15% cleavage at pH 8.7. Linker **6** (**II**) is stable up to pH 8.0, only marginally cleaved at pH 8.7 and cleaved to only approximately 25% at pH 10.5 (Figure 3B). The most pH-labile linker, **5** (**I**) (Figure 3A), was fully cleaved at pH 10.5 after 10 h and cleaved to approximately 70% at pH 8.7 after 48 h. However, **5** (**I**) was still relatively stable up to pH 8 (with approximately 10% cleavage after 24 h).

Based on the above described stabilities of the linkers, we selected linker **6** (**II**) for further characterization because its stability is optimal for the purposes described in the Introduction; sufficient lability in 0.1 M NaOH enabling mild cleavage of compounds but high stability up to pH 8.0, enabling biological applications. Therefore, we tested the stability of linker **6** (**II**) in aqueous ammonia (Figure 3F). The advantage of cleavage with aqueous ammonia is that it can be easily evaporated without any contamination by salts, as is the case for aqueous NaOH. The experiment showed that aqueous ammonia can cleave the peptide from linker **6** (**II**) in a time-dependent manner after 24 h at room temperature. MS analysis after RP-HPLC isolation of two peaks (Figure 4) revealed two products: a C-terminal carboxamide (peptide **31**, entry 5, carboxamide, approximately 75%, HRMS-ESI calculated for $C_{31}H_{49}O_{10}N_9Na$ $[M+Na]^+$730.3495, found 730.3502) and C-terminal carboxylic acid (peptide **31**, entry 5, carboxylic acid, approximately 25%, HRMS-ESI calculated for $C_{31}H_{49}O_{11}N_8$ $[M+H]^+$709.3515, found 709.3511). The lower yield of peptides cleaved with ammonia compared to that cleaved with 0.1 M NaOH (Figure 3F) could be simply caused by a difference in the amounts of wet resin used for the stability experiments.

Conclusion

In conclusion, we have presented a systematic study in which we prepared and characterized the properties of five new acid-stable and diketopiperazine formation-resistant ester linkers, **5** (**I**), **6** (**IV**), **12** (**IV**) and **13** (**V**), and a previously prepared linker, **7** (**III**). The limited solubility of new linker **14** (**IV**) precluded its further evaluation. The main features of these linkers can be summarized in the following points. (i) Linkers **5** (**I**), **6** (**IV**), **7** (**III**), **12** (**IV**) and **13** (**V**) can be easily prepared by a straightforward 3-step procedure on a multigram scale and in high yields. (ii) All the linkers can be easily and effectively attached to TentaGel S NH_2 resin and are fully compatible with Fmoc/*t*Bu protocol solid-phase peptide synthesis protocols, and the ester bonds of all the linkers are stable in 20% piperidine and in concentrated TFA. (iv) Model peptides synthesized on all investigated linkers provided crude products with high purities and high yields. (v) The relative pH stabilities of the linkers in aqueous buffers was as follows: **13** (**V**) > **12** (**IV**) = **7** (**III**) > **6** (**II**) > 5 (**I**), with linker **13** (**V**) being the most stable. This stability order is consistent with the chemical reactivities of the respective ester groups. (vi) All the linkers can be effectively cleaved with 0.1 M NaOH. (vi) All the linkers, except **5** (**I**), which is the most base-labile, are stable up to pH 7.5 in water, and linker **6** (**II**) is stable up to pH 8. This allows their use in the testing of immobilized peptides under standard biological conditions. (vii) We showed that the best linker, **6** (**II**), can also be cleaved with aqueous ammonia, which allows the mild release of a peptide from a resin without any contaminating salts. These properties of the linkers prepared in this study, and especially of linker **6** (**II**), make them useful for solid-phase peptide synthesis when the stable on-resin immobilization of peptides, the testing of their biological properties in aqueous buffers at neutral pH and finally, their release from the solid support under non-destructive conditions are necessary.

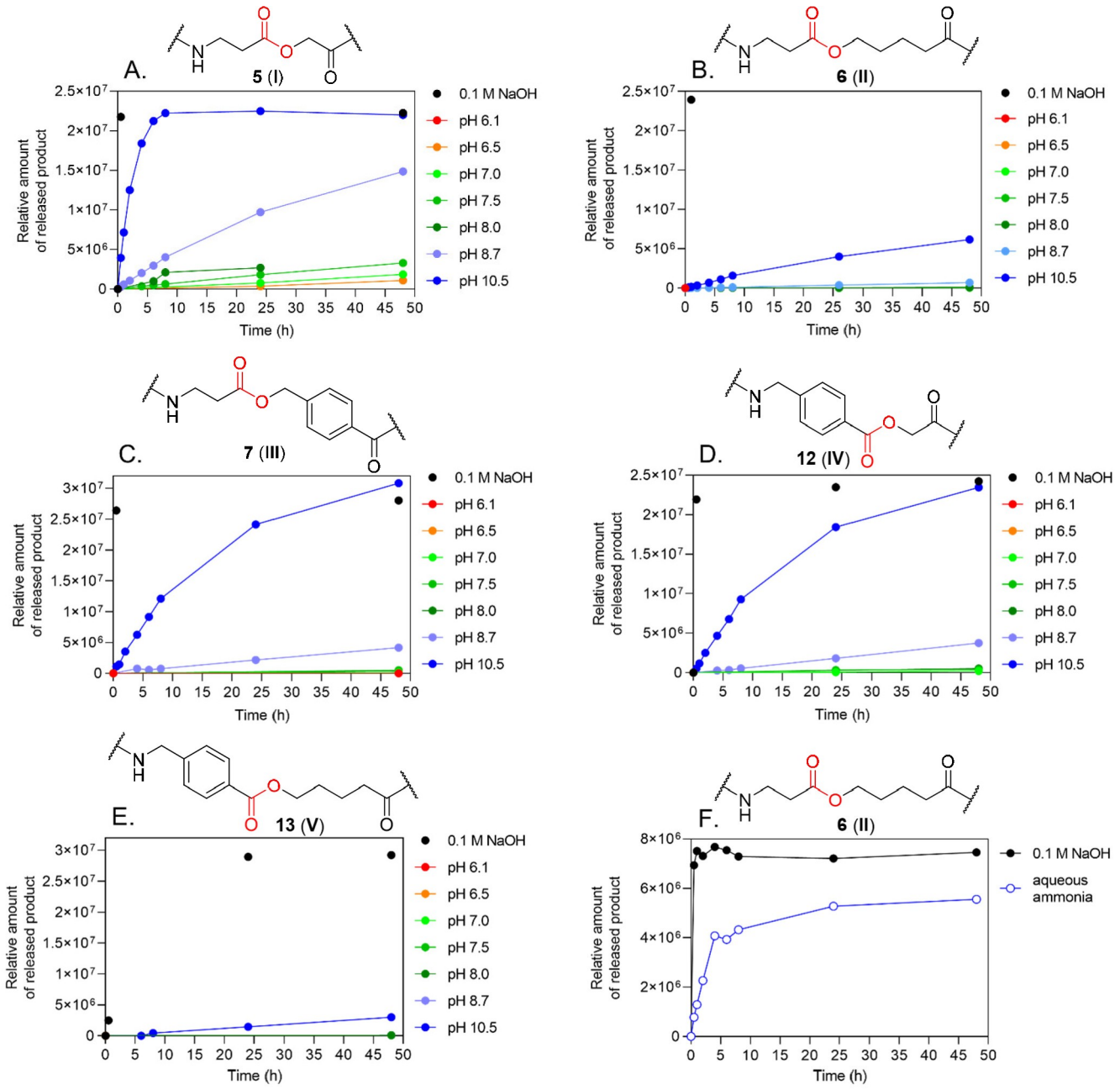


Figure 3. Time-, pH- and solvent-dependent release of Tyr-Ala-Lys-Gly-Glu-Ala-β-Ala peptide attached to TentaGel S NH_2 resins through linkers **5** (**I**) (in A), **6** (**II**) (in B and F), **7** (**III**) (in C), **12** (**IV**) (in D) or **13** (**V**) (in E). The following solvents were used: 50 mM citrate/NaOH at pH 6.1 (red); 50 mM potassium phosphate at pH 6.5 (orange), 7.0 (light green), 7.5 (green) and 8.0 (dark green); 200 mM sodium bicarbonate/sodium carbonate at pH 8.7 (light blue); 50 mM glycine/NaOH at pH 10.5 (dark blue); 0.1 M aqueous NaOH (black) and aqueous ammonia (blue circle), based on the integration of two peaks, see text. The chemical structures of linkers with cleavable ester bonds highlighted in red are also shown.

Experimental Section

General Chemistry

Unless otherwise stated, the reagents and solvents used in this study were obtained from commercial suppliers (Sigma-Aldrich, Fluka, and Merck) and were used without purification. If needed, some solvents were distilled at 50 °C and 2 kPa, and the products were dried over phosphorus pentoxide at rt and 13 Pa. TLC was performed on silica gel-coated aluminum plates (Fluka). The compounds were visualized by exposure to UV light at 254 nm, by ninhydrin spraying (stains amines dark blue), and by spraying with a 1% (v/v) ethanolic solution of 4-(4-nitrobenzyl)pyridine followed by heating and treating with gaseous ammonia (stains esters or alkylating compounds blue). Flash chromatography purifications were carried out on silica gel (40–63 μm, Fluka).

Analytical RP-HPLC was carried out using Method 1 at a flow rate of 1 ml/min on a C8 column (Macherey-Nagel 120–5 C8; 250×4 mm, 5 μm) using the following gradient: t = 0 min (20% B), t = 30 min (100% B), t = 31 min (20% B), or using Method 2 on a C18 column

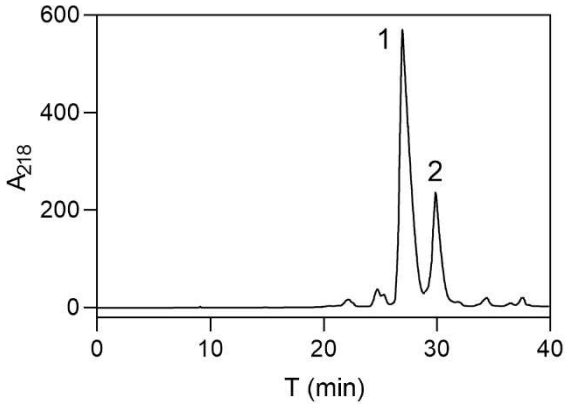


Figure 4. HPLC profile of aqueous ammonia cleavage solutions combined from Tyr-Ala-Lys-Gly-Glu-Ala-β-Ala-linker **6** (**II**)-resin. Chromatographic separation was performed on a preparative C18 column (Watrex Nucleosil 120-5C18, 250×21 mm) using a linear gradient (v/v) of acetonitrile in water with 0.1% TFA (v/v) (0 min–1.6% CH_3CN, 30 min–16% CH_3CN at 9 ml/min). The chromatograms were obtained at 276 nm. The compound that eluted in peak 1 was identified as peptide **31** (entry 5) carboxamide, and the compound that eluted in peak 2 was identified as peptide **31** (entry 5) with a free C-terminal carboxylic group.

(Nucleosil 120 C18; 250×4 mm) using the following gradient: t = 0 min (0% B), t = 30 min (60% B), t = 31 min (0% B). Preparative RP-HPLC was carried out using Method 3 on a C4 column (Grace Vydac C4 214TP; 250×22 mm, 10–15 μm) at a flow rate of 9 ml/min. The following gradient was used: t = 0 min (10% B), t = 30 min (100% B), and t = 31 min (10% B). In all cases, solvent A was 0.1% TFA and solvent B: 80% CH3CN, 0.1% TFA. The eluted compounds were detected at 218 nm.

Melting points were determined on a Boetius block and are uncorrected. 1H and 13 C NMR spectra were acquired on a Bruker AVANCE-600 spectrometer (1H at 600.13 MHz, 13 C at 150.9 MHz) in CDCl3 or DMSO-d6 at 300 K. The 2D H–H COSY, 2D H–C HSQC and 2D H–C HMBC spectra were recorded and used for the structural assignment of proton and carbon signals. IR spectra were recorded on a Bruker IFS 55 Equinox apparatus. HRMS data were obtained on an FTMS mass spectrometer LTQ-orbitrap XL (Thermo Fisher, Bremen, Germany) in electrospray ionization mode, or in the case of HRMS (EI), data were acquired on a GCT Premier (Waters). The purity of each target compound was confirmed by elemental analysis (C, H, N), and the results agreed with the calculated values within 0.3%. The determination of C, H, and N in solid samples was performed using an automatic PE 24000 series II CHNS/O analyzer.

Synthetic details and analytical data for compounds **1**–**30** and methodology for the solid-phase synthesis of model peptides are provided in the Supporting Information.

Determination of the stability of the linkers

Linkers **5** (**I**), **6** (**II**), **7** (**III**), **12** (**IV**) or **13** (**V**) were separately attached to TentaGel S NH_2 resin (Rapp Polymere Gmbh, 130 μm, 0.27 mmol/g), and the same peptide sequence (Tyr-Ala-Lys-Gly-Glu-Ala) were synthesized on each of the linkers as described above. After completion of the synthesis, the resins were thoroughly washed with deionized water until the rinsate was neutral with pH indicator paper. The excess water was removed. Approximately 100 mg of the wet resin was weighed to test tubes. Then, 1.5 ml of the selected buffer (see above in the Results section), aqueous 0.1 M NaOH or aqueous ammonia (24% w/w) was added to the resin. The test tubes were shaken at room temperature. Aliquots (15 μl) of the supernatant were taken after various time periods and diluted with 185 μl of water. The samples (100 μl) were the analyzed by RP HPLC on a C18 analytical column (Watrex Nucleosil 120-5C18, 250× 4 mm) using a linear gradient (v/v) of acetonitrile in water with 0.1% TFA (v/v) (0 min–1.6% CH_3CN, 30 min–16% CH_3CN at 1 ml/min). The chromatograms were evaluated at 218 nm, and the peaks of the products were integrated.

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Conflict of Interest

The authors declare no conflict of interest.

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