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Synthesis and comparative properties of two amide-generating resin linkers for use in solid phase peptide synthesis‡

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Well-characterized resins of high purity are critical for effective solid phase peptide synthesis (SPPS). The quality of commercial (4-methyl)benzhydrylamine-resin (MBHA-resin), used for the synthesis of peptide amides, is not consistent and residual ketone functionalities are frequently present. Such ketone or aldehyde impurities lead to the formation of acylation-resistant deletion peptides in SPPS. To avoid these undesirable side reactions, we have optimized the preparation of two amide-generating linkers, which, in combination with aminomethyl-resin prepared directly from polystyrene resin, serve as alternatives to MBHA-resin for peptide amide synthesis. Then we have explored their comparative properties in SPPS. Use of sonication in reductive amination facilitated the synthesis of both benzhydrylamine (BHA) and MBHA linkers.

Supporting information may be found in the online version of this article

Keywords: solid phase peptide synthesis; MBHA resin; peptide amide; peptide thioester

Introduction

The nature and amount of resin-bound reactive functional groups plays a critical role in solid phase peptide synthesis [1]. High purity peptides of defined chemical composition can only be synthesized on stable, well-defined functionalized resins free of extraneous reactive functionality [2]. Amide-generating (4-methyl)benzhydrylamine (MBHA) resin [3] (Figure 1, resin **1**) can be particularly problematic: properly prepared, such resins give good results; however, the quality of commercial MBHA-resins, like other commercial resins for use in SPPS [4,5], is not consistent. Like all functionalized solid phase synthesis resins, MBHA-resins are difficult to characterize and cannot be purified.

Results and Discussion

Recently, we had occasion to synthesize the model peptide YGGFL amide [(Leu5)enkephalin amide] on commercial MBHA-resin [6], using Boc chemistry '*in situ* neutralization' SPPS [7]. The purity of the crude peptide product was unacceptable; there were extensive deletion products including YGFL amide and YGGF amide (Figure 2(a)).

Using an identical SPPS synthetic protocol, the peptide YGGFL acid was synthesized in high purity (data not shown) on Boc-Leucine-4-OCH_2Pam-resin (Boc-Leucine acyl-4-(oxymethyl)phenylacetamidomethyl-resin) (Figure 1, resin **3**) prepared from Boc-Leucine-(4-carboxymethyl)benzyl ester and aminomethyl-resin (Figure 1, resin **2**) made directly from polystyrene resin according to published procedures [8]. We concluded that the poor quality of commercial MBHA-resin had caused the low purity of the original peptide products. IR spectroscopy examination of the starting MBHA-resin showed the presence of carbonyl moieties (C=O stretch peaks, 1700 cm^{-1}). Conventional MBHA-resin is prepared by Friedel–Crafts acylation of polystyrene resin with 4-methylbenzoyl chloride, followed by reductive amination of the resulting benzophenone [3]. Incomplete reduction of the benzophenone imine or oxime would give rise to residual resin-bound ketone moieties. Formation of deletion peptides in Boc chemistry SPPS has been attributed to the formation of acylation-resistant Schiff's base compounds involving residual aldehyde or ketone functionalities on the resin with the alpha-amino group of a resin-bound peptide at each stage of SPPS [2].

The preparation by SPPS of high-purity peptides, peptide amides, and peptide-thioesters of 30–50 amino acids is essential for chemical protein synthesis by modern chemical ligation methods [9]. Well-characterized amide-generating resins as an alternative to MBHA-resin would be useful for the unambiguous synthesis of peptide-thioesters [10]. We set out to make such a well-defined amide-generating resin by a route that would preclude the presence of residual ketone functionalities. Our starting point was aminomethyl-resin made directly from polystyrene resin according to published procedures that not only avoid the potential introduction of aldehyde or ketone functionalities but which also chemically scavenge any such functionalities that may be present in the starting polystyrene resin [2,8,11]. This aminomethyl-resin was used in combination with a well-characterized, high-purity amide-generating linker prepared by solution chemistry (Figure 3, linker **4** and linker **5**). The Boc-(4-OCH_2COOH)benzhydrylamine (BHA) linker of Matsueda (Figure 3, linker **4**) [12] is well designed, with a carboxylic acid that can be linked to aminomethyl-resin;

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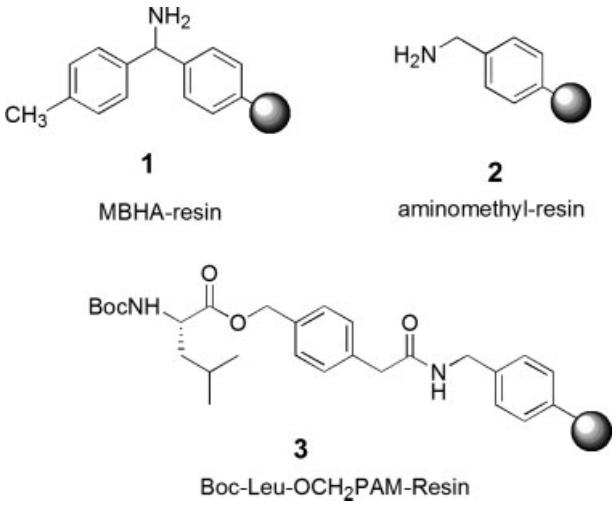

Figure 1. Chemical structures of (a) MBHA-resin **1**; (b) aminomethyl-resin **2**; and (c) Boc-Leucine-4-OCH_2-Pam-resin **3**.

removal of the Boc group gives a substituted benzhydrylamine-resin which can be reacted with any desired *C*-terminal amino acid as the first step in a solid phase synthesis.

Initial attempts to make Matsueda's BHA linker following the protocol described in the original paper were unsuccessful [12]. In our hands, the synthesis was awkward and failed to reproducibly give good yields. The key step for the synthesis is reductive amination of the *p*-hydroxybenzophenone oxime. The original reaction conditions used a large excess of zinc dust at 50 °C in a mixture of concentrated ammonium hydroxide and ethanol for more than 27 h. However, the reaction was slow and was not clean, giving less than 50% conversion after 24 h. Further crystallization from ether and ethanol gave poor yields.

We found that ethanol was not necessary for dissolving *p*-hydroxybenzophenone oxime. The oxime could be dissolved just in ammonium hydroxide aqueous solution. And in order to enhance the speed of the reduction, we employed sonication to make a fresh zinc surface. The resulting reaction was clean and efficient; it gave almost full conversion after 6 h. In addition, the workup was very easy. On adjusting the solution to pH 8 in an ice-water bath, the product precipitated as a white solid. After filtration, washing with water, and drying, the desired (4-OH)benzhydrylamine product was obtained in good yield (>85%) and high purity. Conversion to the Boc-(4-OH)benzhydrylamine, followed by reaction with sodium iodoacetate gave the Matsueda linker (Figure 3, linker **4**) in good yield and high purity.

The obtained Boc-(4-OCH_2COOH)benzhydrylamine linker was coupled to aminomethyl-resin [13], and the resulting BHA-aminomethyl-resin **6** was used for the synthesis of the model peptide YGGFL amide using the Boc chemistry '*in situ* neutralization' SPPS protocols described above. Very high quality crude peptide was obtained (Figure 2(b)). However, HF cleavage of 117 mg of peptide-polystyrene resin gave only 3.3 mg of the YGGFL amide peptide after lyophilization. It seemed to us that the BHA-aminomethyl-resin was too stable to HF cleavage, and that it might be necessary to tune the acid lability of the linker by addition of a methyl group to the benzene ring (Figure 3, linker **5**), in order to increase its acid lability and improve HF cleavage yields. The stability to repetitive TFA treatment of the modified MBHA linker as peptidyl-MBHA aminomethyl-resin would also need to be measured.

Table 1. Comparative peptidyl-resin weights for syntheses on BHA-aminomethyl-resin and MBHA-aminomethyl-resin

Peptide	Scale (mmol)[a]	Calculated weight of peptide-resin (mg)	Weight of peptide resin for BHA-aminomethyl-resin (mg)	Weight of peptide resin for MBHA- aminomethyl-resin (mg)
YGGFL amide	0.2	422	328.3	300.8
LFPGFL amide	0.2	407	305.1	286.8
CFRANK amide	0.2	501	351.8	293.3
Thioester LYRAGXA amide[b]	0.2	489	339.8	321.2
hGHRH(1–29) amide[c]	0.5	3217	2288	1684

[a] Aminomethyl-resin loading 0.98 mmol g^{-1}.
[b] X = SCH_2CH_2CO.
[c] hGHRH, human growth hormone-releasing hormone.

Table 2. Comparative yields of peptides synthesized on BHA-aminomethyl-resin and MBHA- aminomethyl-resin

Peptide	Resin cleaved HF (mg)[a]	Weight of crude peptide cleaved from BHA-aminomethyl-resin (mg)	Weight of crude peptide cleaved from MBHA-aminomethyl-resin (mg)
YGGFL amide	200	5.6	18.3
LFPGFL amide	200	17.8	31.4
CFRANK amide	200	11.7	34.8
thioester LYRAGXA amide[b]	200	17.5	29.4
hGHRH(1–29) amide[c]	500	184.8	228.1

[a] Peptide-resin cleavage by HF was carried out at 0 °C for 1 h using 10 ml HF and 500 ul p-cresol.
[b] X = SCH_2CH_2CO.
[c] hGHRH, human growth hormone-releasing hormone.

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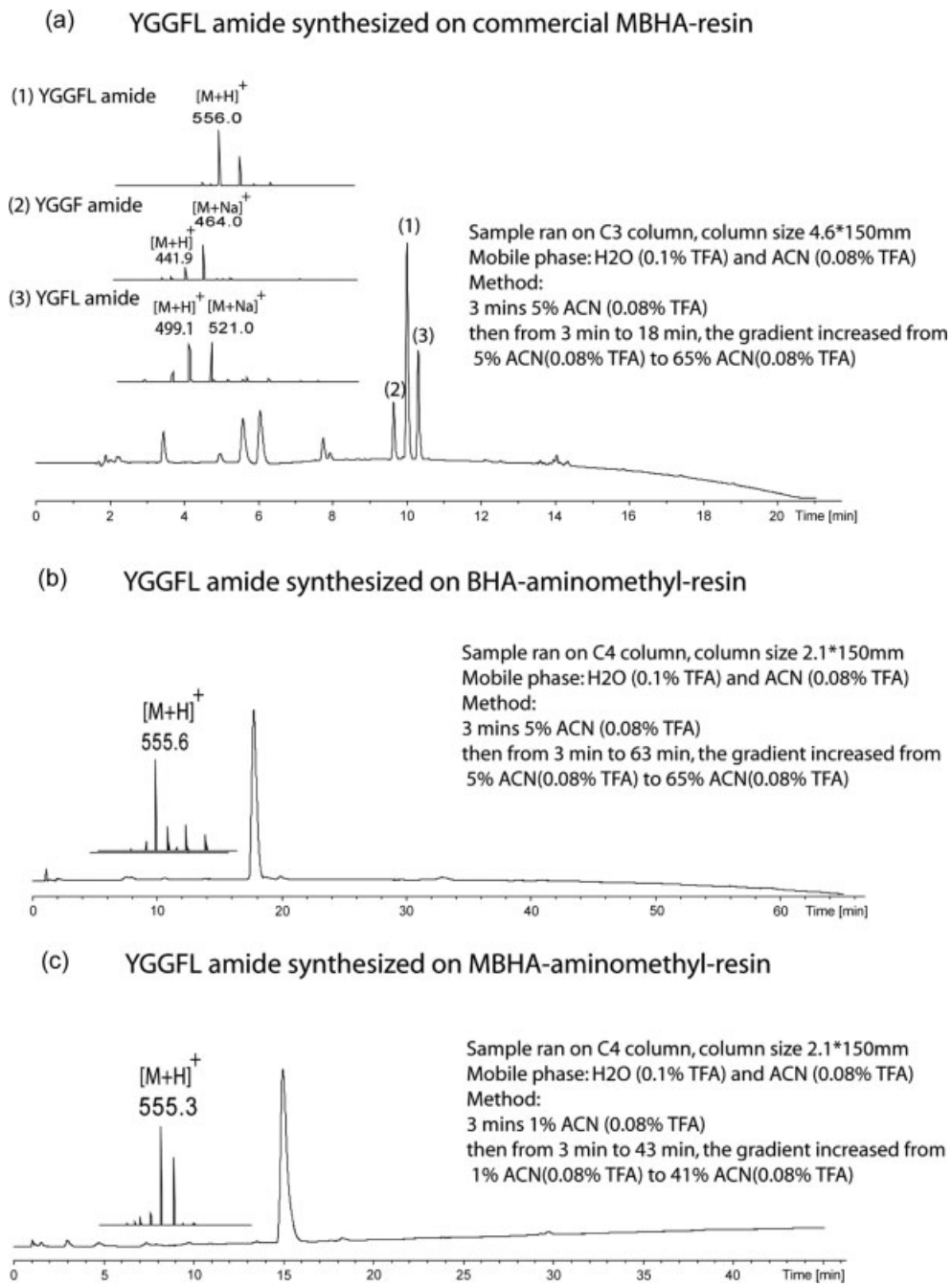

Figure 2. (a) Synthesis of YGGFL amide on commercial MBHA-resin. (b) Synthesis of YGGFL amide on homemade BHA-aminomethyl-resin. (c) Synthesis of YGGFL amide on homemade MBHA-aminomethyl-resin. Note that in (b) and (c) slightly different, ore highly resolving gradients were used.

Synthesis of MBHA aminomethyl-resin is shown in Figure 4. 4-Methoxy-benzoyl chloride **8** reacted with toluene to give 4-methoxy-4′-methyl-benzophenone **9**. Then BBr_3 was used to deprotect methoxy group to give 4-hydroxy-4′-methyl-benzophenone **10** [14]. Sonication-assisted reductive amination of oxime **11**, followed by Boc protection of the resulting amino group of **12**, and reaction with sodium iodoacetate gave MBHA linker **5**. MBHA linker was coupled to aminomethyl-resin by di-isopropylcarbodiimide activation, to afford MBHA-aminomethyl-resin **7**.

We used both BHA-aminomethyl-resin and MBHA-amino-methyl-resin for comparative syntheses of a series of model peptides and hGHRH(1–29)(human growth hormone-releasing hormone) amide. Weights of peptide-resins after SPPS assembly of the protected peptide chains are shown in Table 1. The yields of protected peptide-resins were greater for BHA-aminomethyl-resin than for the MBHA-aminomethyl-resin, suggesting that the MBHA linker was labile to the conditions of Boc chemistry SPPS chain assembly (presumably the repetitive TFA treatments). The weights of crude peptides obtained after HF cleavage of the peptide-resins are shown in Table 2. Peptides synthesized on MBHA-aminomethyl-resin gave higher crude yields than peptides made on BHA-aminomethyl-resin, for an equal weight of peptide-resin cleaved.

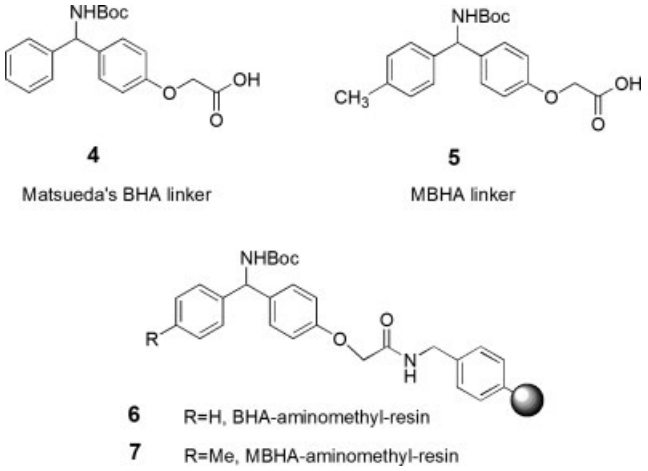

Figure 3. Structures of (a) Matsueda's linker **4**; (b) the modified MBHA linker **5**; (c) BHA- aminomethyl-resin **6**; and (d) MBHA-aminomethyl-resin **7**.

The stability to TFA of the peptidyl-resins was examined for both the BHA-aminomethyl-resin and MBHA-aminomethyl-resin. This was done by putting peptide resin in neat TFA and using HPLC to monitor amounts of peptide cleaved from resin in TFA. The results for LFPGFL amide- were that peptidyl-MBHA-aminomethyl-resin lost 2% peptide per hour in neat TFA, and peptidyl-BHA-aminomethyl-resin lost 0.9% peptide per hour in neat TFA.

Analytic HPLC-electrospray mass spectrometry (LCMS) was used to evaluate the comparative purity of the crude peptide thioester and peptide amide products from both the BHA-aminomethyl-resin and MBHA-aminomethyl-resin (Figure 5). Both MBHA-aminomethyl-resin and BHA-aminomethyl-resin gave high-quality crude peptide products. Full experimental details of the synthesis of the resin linkers and peptide syntheses can be found in the Supporting Information.

Conclusion

We have developed robust syntheses of two resin linkers for making peptide amides on aminomethyl-{polystyrene resin} free of extraneous functional groups. Both MBHA-aminomethyl-resin and BHA-aminomethyl-resin give crude peptide products of high purity, reflecting the unambiguous protocols used for the syntheses of the two resins. MBHA-aminomethyl-resin gave higher yields of crude peptide product for short peptides. For the 29-residue hGHRH amide peptide, the two resins gave comparable overall yields of high-quality crude peptide products. It would seem that the BHA-aminomethyl-resin of Matsueda is somewhat too stable to HF, while the MBHA- aminomethyl- resin is too labile to TFA.

Either of these amide-generating resins can be used to make peptide thioesters, cleanly and in good yield, for use in the chemical synthesis of proteins by modern ligation methods. Further fine tuning of acid stability of amide-generating resins will be done in our future research.

Acknowledgements

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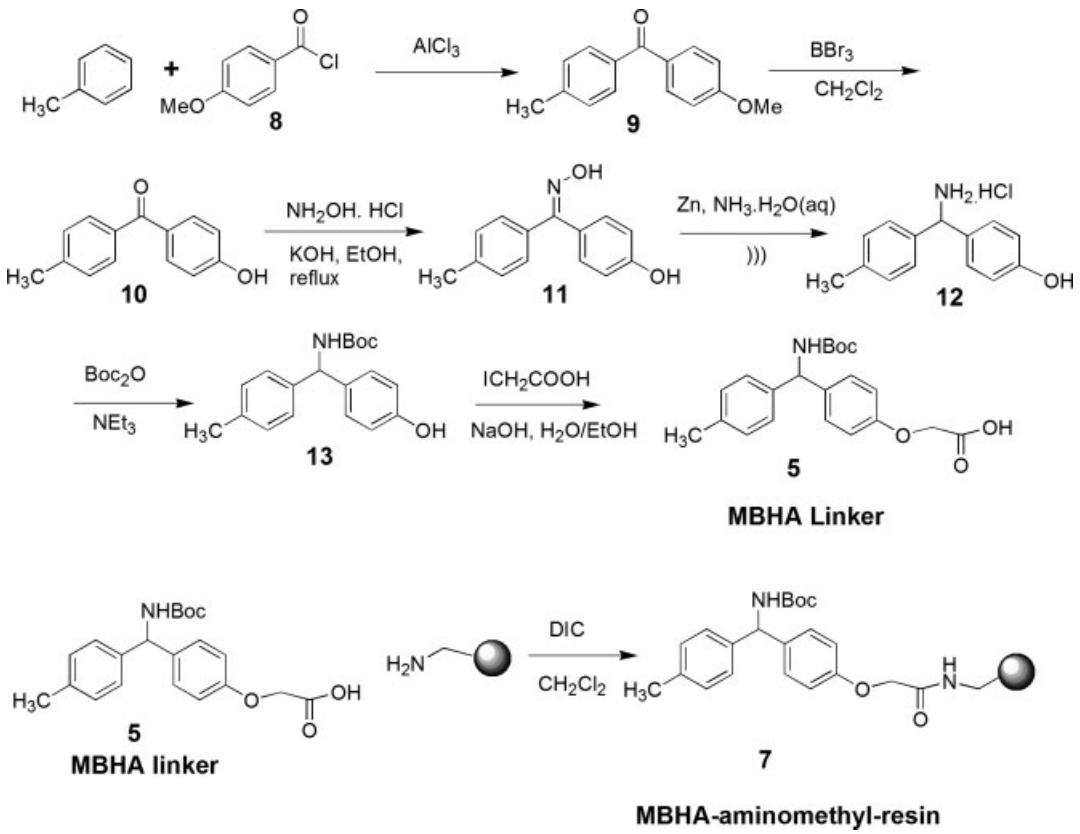

Figure 4. Synthesis of MBHA-aminomethyl-resin **7**, by a variation of the route described by Gaehde and Matsueda [12].

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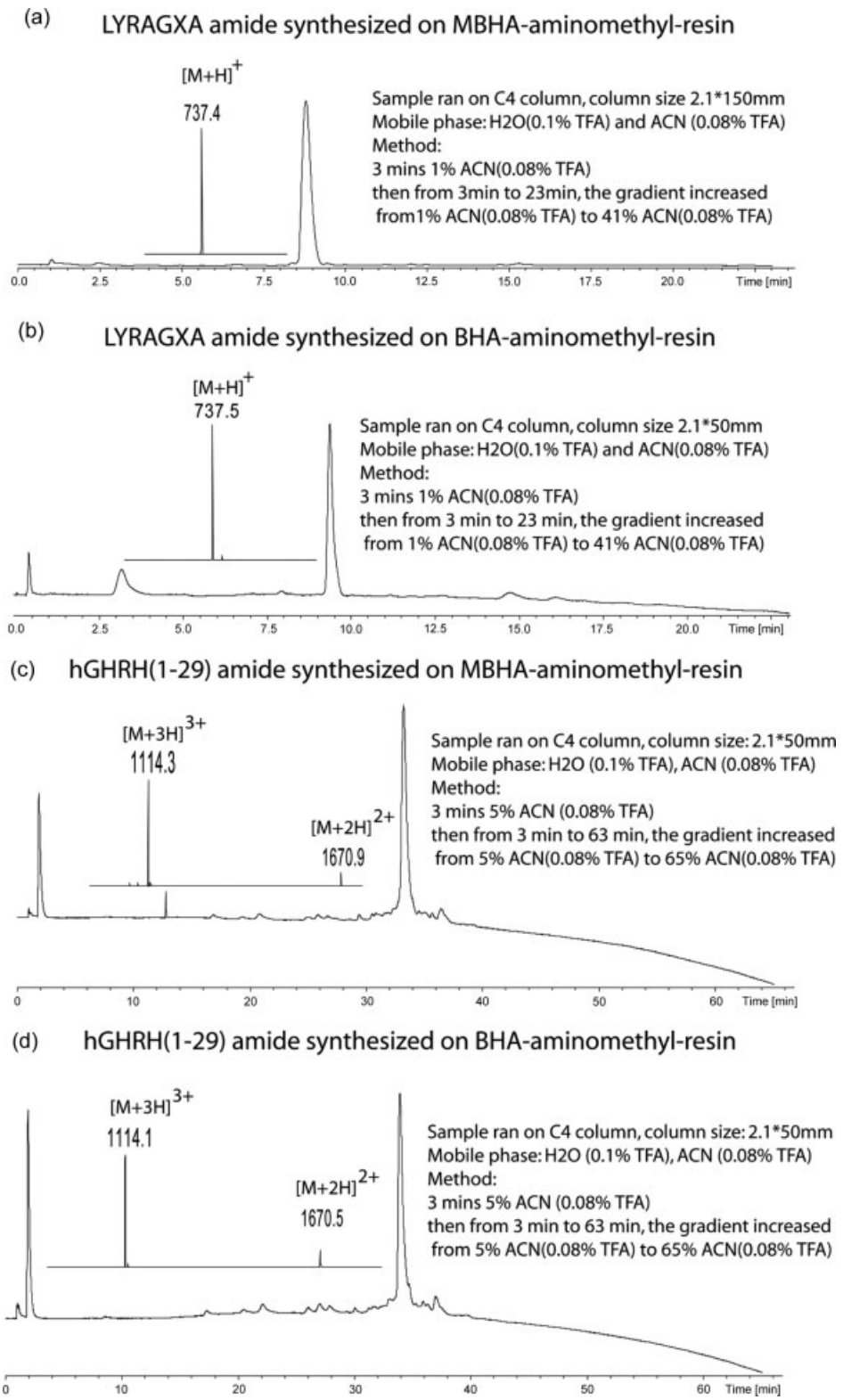

Figure 5. LC-MS data of (a) crude thioester LYRAGXA amide (X = SCH_2CH_2CO) synthesized on MBHA-aminomethyl-resin **7**; (b) crude thioester LYRAGXA amide (X = SCH_2CH_2CO) syntesized on BHA-aminomethyl-resin **6**; (c) crude hGHRH (human growth hormone releasing hormone)(1–29) amide synthesized on MBHA-aminomethyl-resin 7 and (d) crude hGHRH (1–29) amide synthesized on BHA-aminomethyl-resin 6. Different columns were used in (a) and (b).

Supporting information

Supporting information may be found in the online version of this article.

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