Glycopeptides

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Synthesis of the Highly Glycosylated Hydrophilic Motif of Extensins**

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Abstract: *Extensin, the structural motif of plant extracellular matrix proteins, possesses a unique highly glycosylated, hydrophilic, and repeating Ser_1Hyp_4 pentapeptide unit, and has been proposed to include post-translational hydroxylation at proline residue and subsequent oligo-L-arabinosylations at all of the resultant hydroxyprolines as well as galactosylation at serine residue. Reported herein is the stereoselective synthesis of one of the highly glycosylated motifs, Ser(Galp$_1$)-Hyp(Araf$_4$)-Hyp(Araf$_4$)-Hyp(Araf$_3$)-Hyp(Araf$_1$). The synthesis has been completed by the application of 2-(naphthyl)methylether-mediated intramolecular aglycon delivery to the stereoselective construction of the Ser(Galp$_1$) and Hyp(Araf$_n$) fragments as the key step, as well as Fmoc solid-phase peptide synthesis for the backbone pentapeptide.*

Hydroxyproline-rich glycoproteins (HRGPs),[1] which are major structural components of plant extracellular matrices, are produced by extensive post-translational modifications of proline residues. They are first hydroxylated by prolyl 4-hydroxylases[2] and resultant hydroxyproline (Hyp) residues are glycosylated by L-arabinofuranosyltransferases (AFT).[3] These modifications are widespread in plants and essential for their developmental processes such as root hair growth.[4] Secreted peptide hormones of plant origin,[5] such as CLV3,[6] are modified in a similar manner. In addition to arabinofuranosylation, glycosylation of serine (Ser) residues has been found in extensins.[7] Glycosylation has been proposed to enhance their conformational rigidity and are important for molecular recognition[8] required for self-assembly of plant cell walls.[9]

Extensins are pivotal components of plant cell wall architectures, and are required for their self-assembly. Extensin monomers are bipartite in nature, thus consisting of hydrophobic and hydrophilic repeating motifs. Crosslinking of highly conserved Tyr-Xaa-Tyr motifs by oxidative coupling of their Tyr residues confers insolubility on extensins.[10,11] In contrast, the most common repeating motif of their hydrophilic region is the Ser-Hyp-Hyp-Hyp-Hyp pentapeptide modified by Ser α-D-galactopyranosylation [Ser(Galp_1)] and Hyp oligo-L-arabinofuranosylation [Hyp(Ara$f$)$_{1\text{-}4}$] (Figure 1).[12,13] The hydrophilic motif of a HRGP related to root hair growth[12] was proposed to consist of, from the N-to-C terminus, Ser(Galp_1), [Hyp(Araf_4)]$_2$, Hyp(Araf_3), and Hyp(Araf_1) residues. In Hyp(Ara$f_{1\text{-}3}$), all glycosides have been found to be β-linked, while Hyp(Araf_4)[14,15] has an α-L-Ara$f$ residue 1→3-linked to the Hyp(Araf_3). In addition to their structures and activities, biosynthetic as well as metabolic processes have been subjects of recent studies. For example, β-L-arabinofuranosidases (AFases)[16] and β-L-AFT[17] have been identified recently. Interestingly, HypBA1, one of the AFases, was indicated to be a cysteine glycosidase.[18]

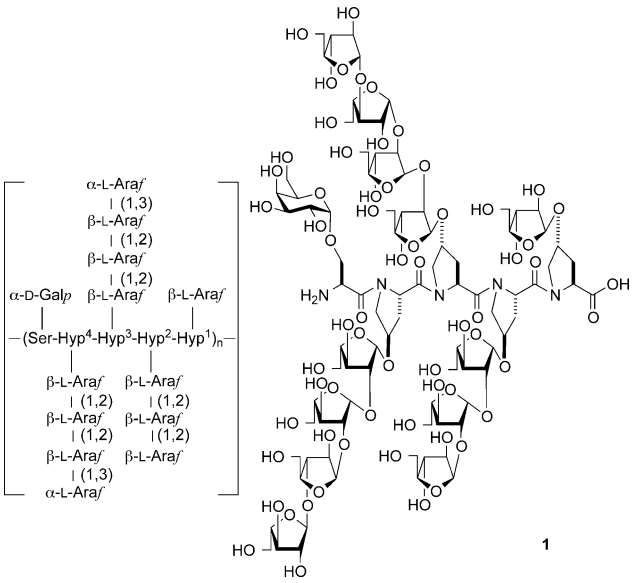

Figure 1. The hydrophilic repeating motif of the extensins **1**.

The hydrophilic motif **1** is synthetically challenging because they are extensively modified by oligosaccharides consisting of consecutive β-Araf*s*. Since stereoselective construction of β-Ara*f* glycosides is difficult to achieve because of its 1,2-*cis* nature, various approaches[19] based on direct[20] or intramolecular[21,22] glycosylation strategies have been examined by targeting extensin structure motifs.[23,24] In contrast, preparation of Ser(Galp_1) is intrinsically more straightforward and has been carried out in a conventional manner.[25]

For the synthesis of 1,2-*cis* glycosides, approaches based on intramolecular aglycon delivery (IAD)[26] have been

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employed successfully. Among a number of variants reported, 2-(naphthyl)methyl (NAP) ether mediated IAD has been shown to be most versatile,[27,28] and applied to the synthesis of CLV3[29] and *p*-nitrophenyl β-L-Ara*f*,[30] a valuable substrate for HypBA1. Herein we report the first synthesis of a hydrophilic repeating motif typical of extensins, Ser(Gal*p*$_1$)-Hyp-(Ara*f*$_4$)-Hyp(Ara*f*$_4$)-Hyp(Ara*f*$_3$)-Hyp(Ara*f*$_1$) (**1**). It features the extensive use of NAP-IAD for all 1,2-*cis* glycosides, including Ser(Gal*p*$_1$) and Hyp(Ara*f*$_n$) ($n = 1, 3, 4$). Subsequent Fmoc solid-phase peptide synthesis (Fmoc-SPPS) was carried out with suppression of diketopiperazine (DKP) formation.

Imaginary disconnection of the target structure led us to design fragments corresponding to Ser(Gal*p*$_1$) (**5**) and Hyp-(Ara*f*$_n$) ($n = 1, 3, 4$; **2**–**4**; Figure 2). Among them, **2** and **3** were prepared as previously reported[29] (Scheme 1). Namely, starting from **10**, oxidative mixed acetal (MA) formation with the donor **11** in the presence of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) was followed by IAD mediated by MeOTf and 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP) to afford the Hyp(Ara*f*$_1$) derivative **12** in a fully stereoselective manner. Then **12** was converted into **2** in a conventional manner.

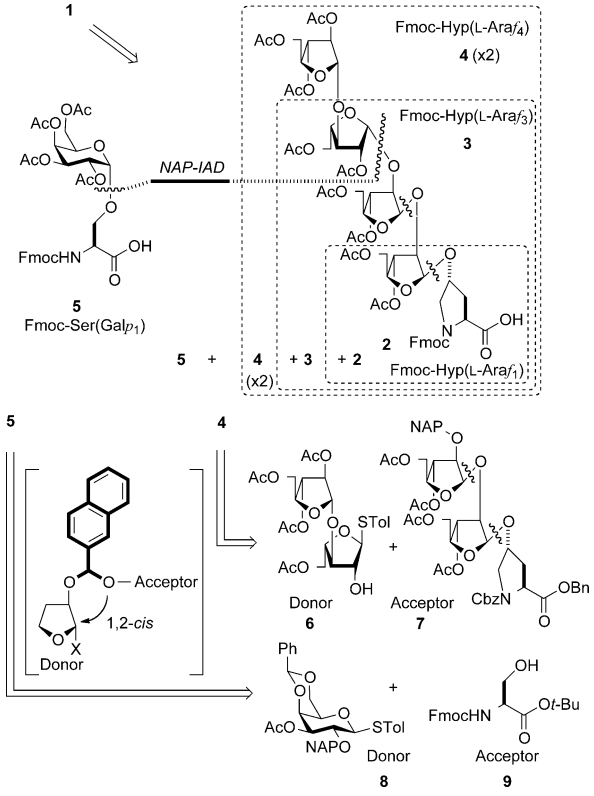
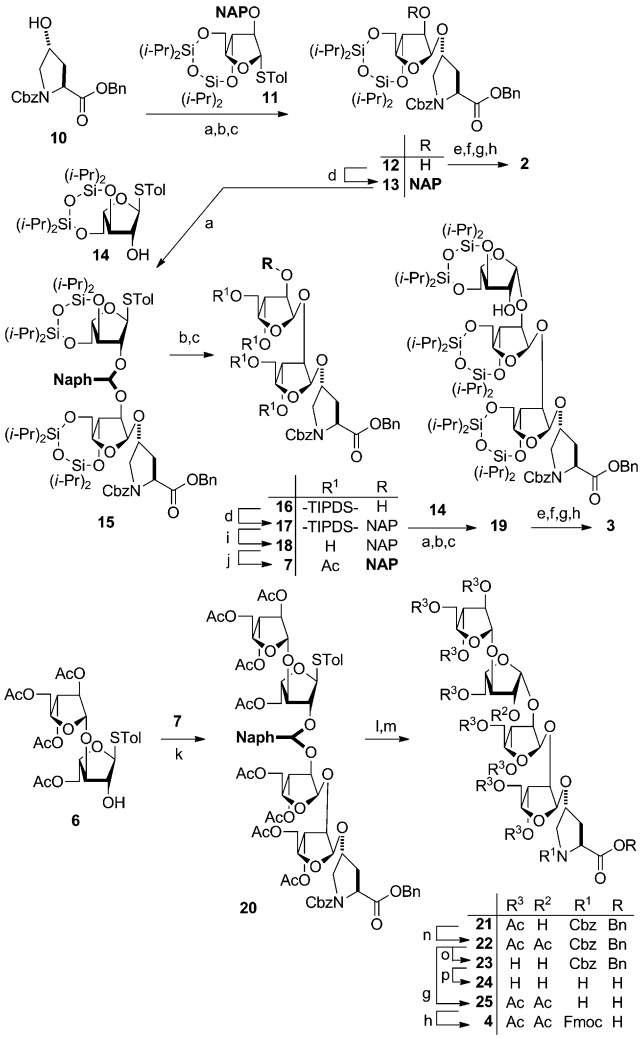


Figure 2. Synthetic plan for (oligo)saccharyl amino acid fragments of **1**. Cbz = benzyloxycarbonyl, Fmoc = 9-fluorenylmethyloxycarbonyl, Tol = 4-methylphenyl.



Scheme 1. Stereoselective synthesis of Hyp(Ara*f*$_{1\text{-}4}$) derivatives. a) DDQ, 4Å M.S., DCE, RT; b) MeOTf, DTBMP, 4Å M.S., DCE, 40°C, 48 h; c) TFA, $CHCl_3$, 0°C, 2 h, 70% (**12** from **10**), 73% (**16** from **13**), 51% (**19** from **17**); d) NAPBr, NaH, TBAI, DMF, −20°C, 6 h, 82% (**13**), 79% (**17**); e) TBAF, pyr/THF, 0°C, 1 h; f) Ac_2O, py, RT, 3 h; g) H_2, $Pd(OH)_2$, EtOAc/EtOH (2:1), RT, 8–16 h; h) FmocCl, DIPEA, CH_2Cl_2, RT, 5 h, 72% (**2** from **12**), 70% (**3** from **19**), 70% (**4** from **26**); i) TBAF, THF, 0°C, 1 h; j) Ac_2O, py, 89% from **17**; k) DDQ, 4Å M.S., DCE, RT, 74%; l) MeOTf, DTBMP, 4Å M.S., DCE, 40°C, 48 h; m) TFA, $CHCl_3$, 0°C, 0.5 h; n) Ac_2O, py, 84% from **20**; o) 0.1 M NaOH, MeOH, 0°C, 4 h; p) H_2, $Pd(OH)_2$, MeOH/H_2O/HOAc (30:10:1), RT, 15 h, 70% from **22**. M.S. = molecular seives, DCE = $(CH_2Cl)_2$, DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, DIPEA = $(iPr)_2NEt$, DTBMP = 2,6-di-*tert*-butyl-4-methylpyridine, Naph = 2-naphthyl, py = pyridine, TBAF = $(nBu)_4NF$, TBAI = $(nBu)_4NI$, Tf = trifluoromethanesulfonyl, TFA = trifluoroacetic acid, TIPDS = tetraisopropyldisiloxanylidene.

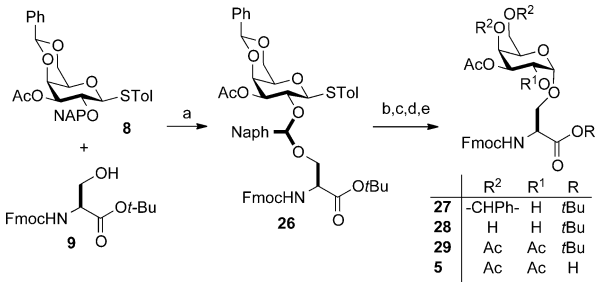
From the preceding study,[29] we learned that in order for the IAD to be most efficient, proper combination of donor and acceptor is critical. Specifically, the use of an NAP-masked acceptor in combination with a 2-*O*-unprotected donor is optimal for the construction of consecutive β-Ara*f* linkages. Accordingly, **12** was converted into the corresponding NAP ether **13**, which was coupled with the donor **14** through the MA **15** under standard NAP-IAD conditions. After conversion of **16** into **17**, chain elongation was carried out in a similar manner to give **3** from **17** through **19**.

For the preparation of the Hyp(Ara*f*$_4$) component **4**, a fragment coupling between the donor **6**[31] and the acceptor **7** was planned (Figure 2). The acceptor **7** was prepared from **17** through desilylation and acetylation of the resultant **18**

(Scheme 1). Coupling of **6** with **7** stereoselectively afforded **21** from the MA **20**, which was converted into **22**. Since, judging from NMR spectra, **22** was a mixture of rotamers, its homogeneity was confirmed after being converted into the free Hyp(Araf_4) **24** from **23**. The compound **22** was then converted into **4** by way of **25**.

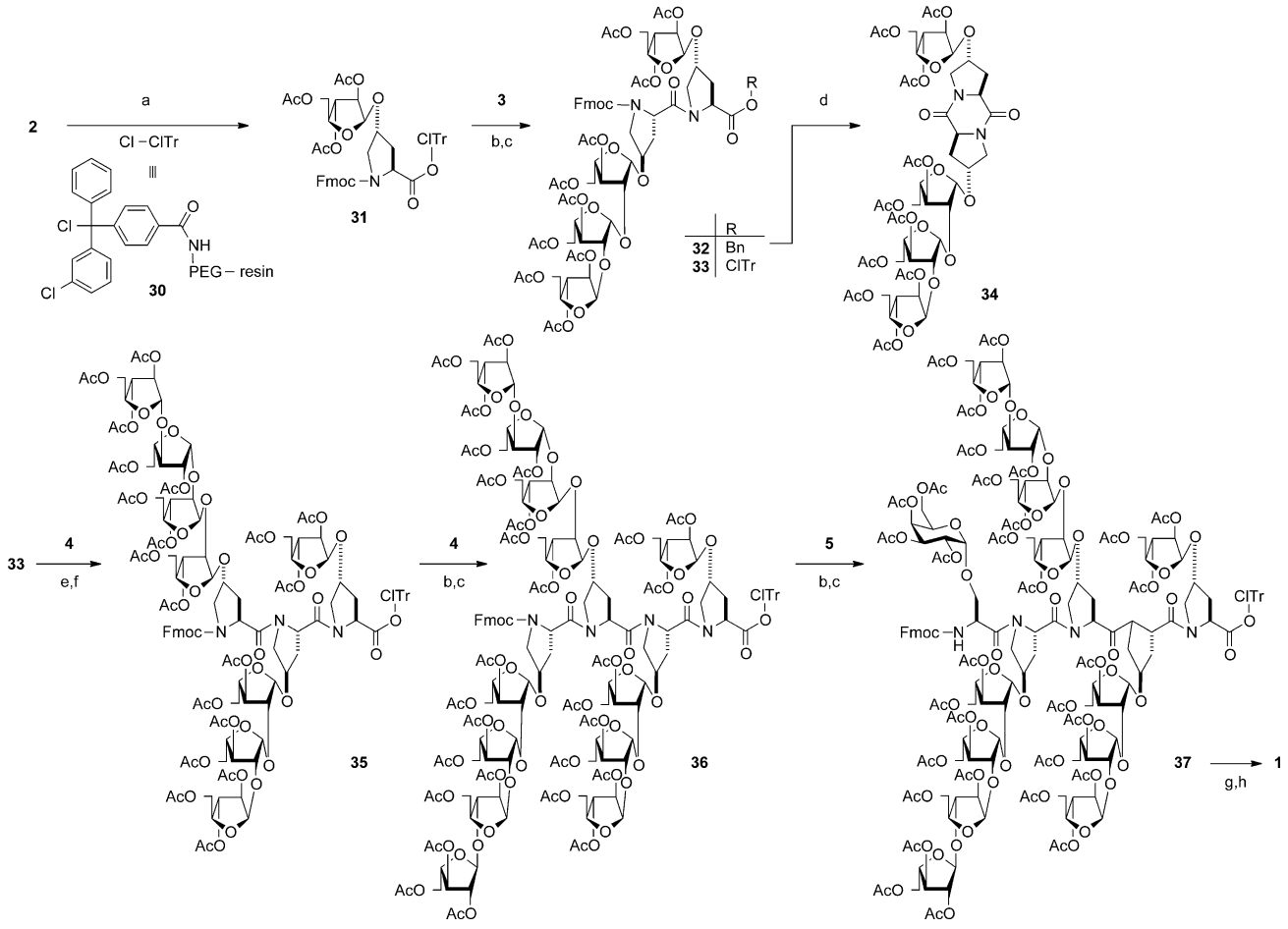
The Fmoc-Ser(α-Galp_1) derivative **5** was synthesized from the NAP-protected donor **8**[31] (Figure 2, Scheme 2), although **5** was also obtained predominantly by direct intermolecular glycosylation.[31] The donor **8** was treated with the Fmoc-Ser-O-*t*Bu **9** under oxidative mixed-acetal-formation conditions to give the MA **26**, which was converted into the expected α-galactosylserine derivative **29** in a highly stereoselective manner after mild acidic work-up (10% TFA in $CHCl_3$ at 0°C) of **27** and *O*-acetylation of the resultant triol **28**. Further acidic removal of the *tert*-butylester of **29** gave the desired Fmoc-Ser(Galp_1) derivative **5**.[25]

We initially planned to conduct solution-phase peptide synthesis using 1-[(1-(cyano-2-ethoxy-2-oxo-ethylideneaminooxy)-dimethylaminomorpholino)]uronium hexafluorophosphate (COMU)[32] as a coupling reagent[29] (Scheme 3). However, not unexpectedly,[33] treatment of the dipeptide derivative **32**[31] with piperidine to remove the Fmoc group



Scheme 2. Stereoselective synthesis of **5**. a) DDQ, 4Å M.S., DCE, RT, 98%; b) MeOTf, DTBMP, 4Å M.S., DCE, 40°C, 48 h; c) 10% TFA, $CHCl_3$, 0°C, 2 h; d) Ac_2O, py, 56% from **30**; e) 20% TFA, $CHCl_3$, RT, 2 h, 84%.

resulted in formation of a significant amount of the DKP **34** (MALDI-TOF MS: $[M+Na]^+$ calc for $C_{50}H_{66}N_2Na_1O_{30}$, 1197.35, found 1197.43). This problem was circumvented by Fmoc-SPPS using the chlorotrityl (ClTr) resin **30**.[34] Namely, after introducing **2**, the resin **31** was subjected to Fmoc removal and COMU coupling with **3** to give the resin-bound dipeptide **33**. Subsequent Fmoc removal was carried out with piperidine in the presence of 1-*O*-hydroxybenzotriazole



Scheme 3. Solid-phase glycopeptide synthesis toward **1**. a) DIPEA, RT, 16 h; b) pip, DMF, RT, 10 min; c) COMU, DIPEA, RT, 16 h; d) pip, DMF, RT, 84%; e) pip, HOBt, DMF, RT, 10 min; f) DIC, HOBt, RT, 16 h; g) TFA/(iPr)$_3$SiH/H_2O (190:5:5), 1 h; h) NaOH, MeOH, 0°C, 21% based on initial loading to ClTr resin. pip = piperidine.

(HOBt)[35] to trap free amino groups, and DIC–HOBt coupling[36] with **4** afforded the tripeptide **35** with suppression of the DKP formation. The entire sequence of **1** was successfully constructed by iterative chain elongation using **4** and **5** to give **37** via **36**. Finally, the resultant pentapeptide was cleaved from the resin and was deacetylated at 0 °C,[37] thus completing the synthesis of **1**. High-resolution ESI-TOF mass data ($[M+H]^+$ calcd for $C_{89}H_{142}N_5O_{64}$, 2304.8011, found 2304.7968) provided evidence to support the target structure.

For structural analysis, we opted to use the CID method for tandem MS-MS analysis, although glycosidic linkages would be cleaved to a larger extent than in ETD/ECD methods. The fragmentations of all sugar moieties one by one was observed to support the presence of all required sugar residues.[31] The structure of **1** was also analyzed by two-dimensional NMR techniques in detail. The corresponding 13 glycosidic linkages were identified in HSQC spectra (Figure 3), and full assignment of the peaks was made at 900 MHz.[31]

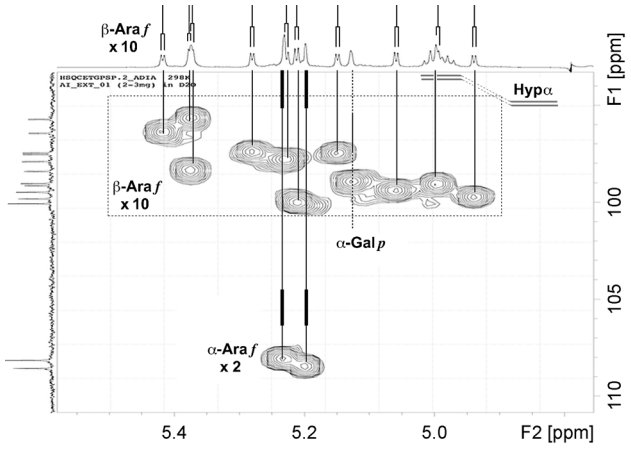


Figure 3. Anomeric reagion of HSQC spectra (900 MHz, D_2O) of **1**.

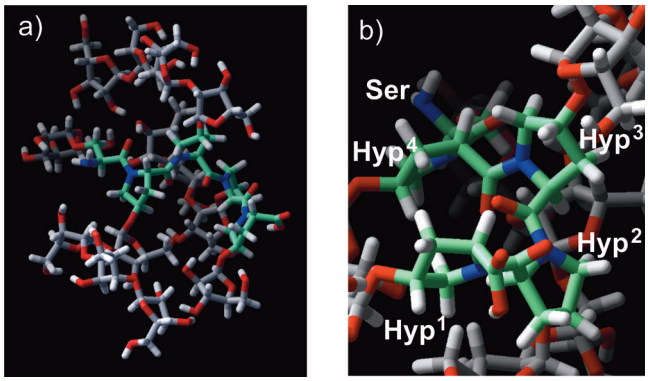


Figure 4. a) Global minimum structure of **1** in H_2O. Carbon atoms of amino acids are colored green. b) Focus on peptides.

The NOESY experiment revealed correlations between all pairs of neighboring α-H(Ser/Hyp) and δ-H_2(Hyp) units, even at 40 °C. Consequently, all Hyps in **1** were suggested to possess *s-trans*-configured amide bonds, thus making the glycopeptide into a left-handed polyPro II helixlike structure[38] (Figure 4), as has been found in nonglycosylated as well as in glycosylated Hyp derivatives.[39,31]

In summary, stereoselective synthesis of Ser(Galp_1)-Hyp-(Araf_4)-Hyp(Araf_4)-Hyp(Araf_3)-Hyp(Araf_1) (**1**) was realized by NAP-IAD for stereoselective constructions of all 1,2-*cis* glycosides containing the fragments Ser(Galp_1) and Hyp-(Araf_n) ($n = 1, 3, 4$). Assembly of the highly glycosylated pentapeptide unit was achieved by Fmoc solid-phase peptide synthesis.

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