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A COMPARISON OF ACID LABILE LINKAGE AGENTS FOR THE SYNTHESIS OF PEPTIDE C-TERMINAL AMIDES

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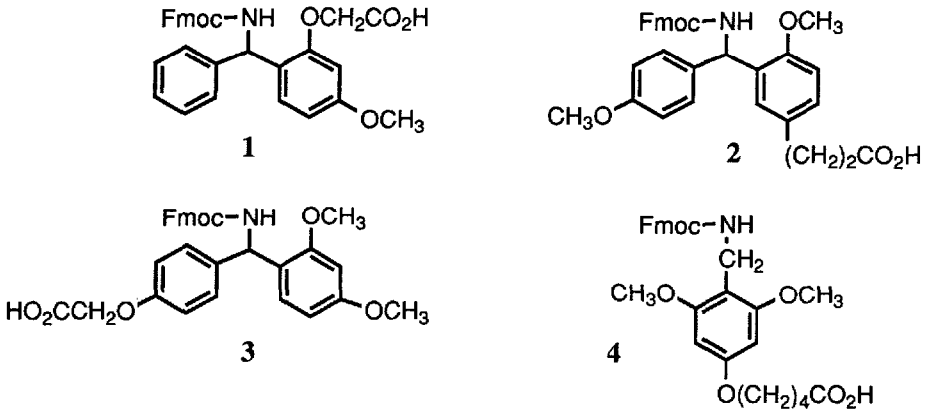
Summary: Substituted benzhydrylamine and benzylamine linkage agents useful for the solid phase peptide synthesis of C-terminal amides were evaluated for their relative lability toward trifluoroacetic acid. The two most reactive linkage agents studied were compared in the synthesis of two different peptide amides by the Nα-9-fluorenylmethyloxycarbonyl protecting group strategy.

Recently the efforts of a number of investigators[1-5] have been directed towards the preparation and use of acid labile linkage agents suitable for the synthesis of peptide C-terminal amides by the N α-9-fluorenylmethyloxycarbonyl (Fmoc)[6] protecting group strategy. These linkage agents are alkyl and/or alkoxy-substituted benzhydrylamines or benzylamines that were designed to allow attachment to amine supports via a carboxyl group and cleavage of peptide amide products from peptide synthesis solid supports under relatively mild acid conditions. Although all such linkage agents reported to date have been used successfully for the synthesis of peptide C-terminal amides, no information was available concerning their relative rates of cleavage. This study was undertaken in order to gain information regarding how the number, nature, and positioning of aromatic ring substituents influence reactivity of the linkages under acidic conditions.

The structures of the linkage agents studied are given in Figure 1. Benzhydrylamine derivative 1 was prepared from commercially available 2-hydroxy-4-methoxybenzophenone (Aldrich) by a straightforward four step procedure. The hydroxyl group of the benzophenone was alkylated with chloroacetic acid in the presence of 6N KOH, NaI and dioxane. The carboxymethylated benzophenone product was converted to its oxime derivative ($HCl \cdot NH_2OH/NEt_3/EtOH$) which was reduced to its benzhydrylamine derivative by the action of zinc dust as reported[7] for a similar benzhydrylamine. The Fmoc-protecting group was introduced in the usual manner (Fmoc-OSu/10% aq. Na_2CO_3/dioxane) to give the desired linkage agent 1 (overall yield ~58%)[8]. Linkage agents 2[5] and 3 were the generous gifts of Professor Haruaki Yajima and Dr. Gareth Priestly respectively. Resin bound linkage agent 4[1] (PAL™-resin) is commercially available from MilliGen/Biosearch.

FIGURE 1



All linkage agents studied were efficiently coupled in dimethylformamide (DMF) with 2.5 equivalents each of linker, dicyclohexylcarbodiimide, and 1-hydroxybenzotriazole (HOBT), to amine-functionalized Pepsyn K™ (polyamide-kieselguhr) resin[9]. After removal of the Fmoc group (20% piperidine in DMF, 10 min) from the resin bound linkage agents, the amino groups were acylated with Fmoc-valine-pentafluorophenyl ester (4 equivalents), HOBT (4 equivalents) in DMF (2 hr, room temperature) to obtain Fmoc-Val-linkage agent-Pepsyn K™ resin derivatives of compounds 1-4. In no case was this acylation observed to be difficult. Fmoc-valine was chosen as the test amino acid for linkage to resins in this manner because its bulky steric properties would presumably make this a more difficult amino acid to couple and cleave.

In order to assess relative reactivity, the Fmoc-Val-linker-resin derivatives of 1-4 were treated with trifluoroacetic acid (TFA) containing 5% by weight phenol, a reagent commonly used for deprotection and cleavage of peptides from resins after synthesis by the Fmoc-strategy. Aliquots of resins were taken at various times and the cleavage reactions quenched by rapid dilution with DMF. After washing (DMF, CH_2Cl_2, ether) and drying of the resins *in vacuo*, the Fmoc-valine remaining attached to the resins was quantitated by a spectrophotometric method[10]. The results are depicted graphically in Figure 2. Half-lifes were approximated from the time course data and are given in Table 1. The relative reactivity observed was 4>3>2>1, with the number and nature of aryl substituents influencing reactivity as expected for the stabilization of a carbonium ion intermediate (alkoxy > alkyl). Interestingly, the trialkoxybenzylamine derivative 4 was significantly more reactive (>2X) than the trialkoxybenzhydrylamine derivative 3. This observed reactivity difference may in part be accounted for by the additional methylene group of 4 separating the carboxyl group of its carboxylated alkoxy substituent from the aromatic ring.

Figure 2

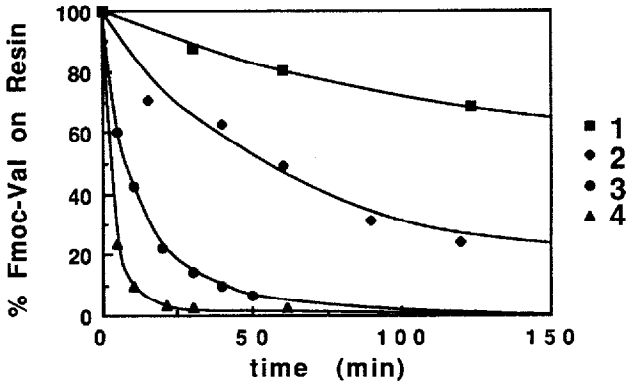


TABLE 1.

Linkage Agent	Half-life (loss of Fmoc-Val-NH_2)
1	~336 min.
2	~ 56 min.
3	~ 9 min.
4	~ 4 min.

In a separate experiment the Fmoc-glycine resin derivative of linkage agent 1 was prepared and subjected to the same cleavage conditions. The substitution of glycine for valine enhanced the rate of cleavage by a factor of ~3. It is unclear from these experiments how peptide chain length and sequence affect the cleavage rate, although it is clear that steric effects arising from the C-terminal amino acid influence the rate of cleavage.

The addition of ethanedithiol (EDT), an efficient carbonium ion scavenger, to the cleavage reagent (TFA:EDT:phenol = 93:5:2) had no effect on the rate of cleavage of the Fmoc-Val resin prepared from linkage agent 1.

From a practical point of view, the results show that linkage agents 3 and 4 are the most satisfactory for the synthesis of peptide C-terminal amides[11]. To compare actual synthesis performance of linkage agents 3 and 4, two peptides, Eledoisin[12] and Neuromedin U-25[12], were synthesized from their Pepsyn K™ resin derivatives. The syntheses were performed on the MilliGen/Biosearch Model 9050 continuous flow automated peptide synthesizer using two reaction columns in series in simultaneous synthesis mode so that both resin derivatives of 3 and 4 were subjected to the same solutions of reagents. Coupling reactions were performed using Fmoc-amino acid-pentafluorophenyl esters (4 equivalents) and HOBT (4 equivalents) in DMF. Cleavage of the peptide

products from the resins was carried out with TFA:phenol (95:5) for 2 hr at room temperature. Yields of isolated crude peptide products were identical (Eledoisin, 91±1%, Neuromedin U-25, 87±1%). HPLC analysis[13] of the crude products showed comparable levels of high purity. The identities of the major products were established by coelution with authentic samples[14].

In conclusion linkage agents 3 and 4 performed equally well for the synthesis of peptide C-terminal amides. The relative reactivity data obtained could be useful in the design of new amide protecting groups and/or linkage agents.

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8. Compound 1 and all synthetic intermediates displayed ^{1}H NMR spectra consistent with their structures.
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10. Spectrophotometric determination based on Fmoc-derived chromophore liberated upon treatment with piperidine (ε_{301} = 7,800 M^{-1} cm^{-1} in 4% piperidine in CH_2Cl_2).
11. Although the Fmoc-Val-linker resin derivative of 3 requires about one hour for >95% cleavage, this is inconsequential in a practical sense since >1 hr exposure to the cleavage reagent is routinely performed to assure complete removal of side-chain protecting groups. The practical utility (rate of cleavage) of linkage agents 1 and 2 may be enhanced by the use of additives such as thioanisole to the cleavage reagent (see Funakoshi et al. ibid.)
12. Eledoisin and Neuromedin U-25 have the sequences pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-amide and Phe-Lys-Val-Asp-Glu-Glu-Phe-Gln-Gly-Pro-Ile-Val-Ser-Gln-Asn-Arg-Arg-Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-amide respectively.
13. HPLC was performed on a Waters Delta-Pak C-18, 100Å, 5um pore, 0.39x15 cm column with a flow rate of 1.0 ml/min at 30°C. Detection was at 220 nm. A linear gradient elution of 6 to 62% acetonitrile in water containing 0.1% TFA was performed.
14. Eledoisin and Neuromedin U-25 were purchased from Bachem Inc., Torrance, CA.

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