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A modified *B*ackbone *A*mide *L*inker (BAL) solid-phase peptide synthesis strategy accommodating prolyl, *N*-alkylamino acyl, or histidyl derivatives at the C-terminus[1]

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Abstract

A new variation of the *B*ackbone *A*mide *L*inker (BAL) approach has been developed for N^{α}-Fmoc solid-phase synthesis (SPS) of C-terminal modified peptides; this provides a convenient route to peptides containing prolyl, *N*-alkylamino acyl, or histidyl derivatives at the C-terminus. To illustrate the principles, several model peptides were prepared in high yields and excellent purities; diketopiperazines and racemized byproducts which otherwise form when alternative procedures are used were absent from the syntheses reported herein. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: backbone amide linker; C-terminal modified peptides; diketopiperazines; histidine; proline; PyBOP; racemization.

Traditional N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) based approaches for the solid-phase synthesis (SPS) of peptide acids generally proceed by activation of the C^{α}-carboxyl group of the C-terminal amino acid residue, and subsequent esterification onto *p*-alkoxybenzyl alcohol functionalized resins[2] in the presence of *N*,*N*-dimethyl-4-aminopyridine (DMAP) as a catalyst. Such methods are prone to loss of the chiral integrity of the amino acid residue esterified.[3] Of the 20 proteinogenic amino acids, histidine is by far the most prone to racemization during activation,[4] even with an array of N^{im}-protecting groups,[2] such as N^{π}-*tert*-butoxymethyl (Bum), N^{τ}-trityl (Trt), and N^{τ}-*tert*-butyloxycarbonyl (Boc). As a separate issue, N^{α}-Fmoc synthesis of peptide acids containing Pro or *N*-alkylamino acids at the C-terminal position, and anchored as the usual *p*-alkoxybenzyl esters, is particularly problematic due to diketopiperazine (DKP) formation at the dipeptidyl resin stage, with concomitant loss of chains from the support.[2] Even when the anchor is created by esterification onto 2-chlorotrityl chloride resin,[5] DKP formation can occur (F. Albericio, unpublished results, 2000).

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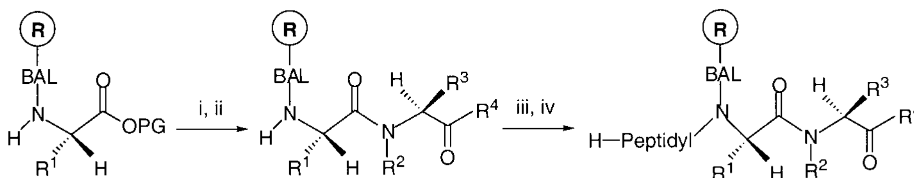
In this communication, we report how our previously described BAL approaches[6,7] can be adapted in a novel manner to overcome the challenges listed in the opening paragraph. Moreover, one need not be restricted to C-terminal peptide *acids*; essentially any C-terminal modification stable to piperidine can be accommodated. Recall that the original BAL approach[6] starts with a reductive amination step involving the eventual C-terminal residue, and is hence unsuitable for incorporation of secondary amines such as prolyl and *N*-alkylamino acyl derivatives. An *extended* BAL approach[7] designed to allow incorporation of relatively non-nucleophilic and/or base-sensitive residues—after completion of peptide chain assembly—is not particularly suited for prolyl or *N*-alkylamino acyl derivatives either, because of the risk for racemization (1.5–3%, or more depending on coupling rate) at the penultimate residue. As for histidine, the original BAL approach does lead to racemization; albeit less than by direct esterification (see Refs. 13 and 15 of this paper). As already mentioned, modest racemization at the penultimate residue is expected by the extended BAL approach.[7]

Our new approach (Scheme 1) consists of: (i) start of SPS by anchoring to the support the eventual penultimate residue, with its C^{α}-carboxyl group protected as an allyl ester or *tert*-butyl ester;[8] (ii) selective removal of the C^{α}-carboxyl protecting group, without cleavage of the BAL anchor;[8] (iii) activation of the C^{α}-carboxyl group using a phosphonium salt,[9] and coupling on the desired C-terminal residue (in this approach, racemization during activation can occur only by a direct enolization mechanism; since the oxazolone mechanism is precluded, essentially no racemization is observed); (iv) acylation of the BAL-anchored amine, using a symmetrical anhydride in CH_2Cl_2–DMF (9:1); (v) peptide chain elongation with standard Fmoc protocols; and (vi) final acidolytic cleavage to release the free peptide into solution.



Scheme 1. SPS of C-terminal modified peptides by the title BAL strategy. The initial reductive amination to anchor H-AA^1-OPG to PALdehyde resin, and the final acidolytic cleavage step, are not shown. (i) PG = allyl; $Pd(PPh_3)_4$ (5 equiv.) in $CHCl_3$–HOAc–NMM (37:2:1) under Ar, 25°C, 2 h; PG = *t*Bu; neat TFA, 25°C, 6×10 min; (ii) H-(R^2)-AA^3-R^4–DIEA–PyBOP (10:20:10) in DMF, 2 h; (iii) introduction of the third AA residue from C-terminal: $(Fmoc\text{-}AA)_2O$ (5 equiv.) in CH_2Cl_2–DMF (9:1), 2×2 h; (iv) standard cycles of Fmoc chemistry

The first target to illustrate the new strategy was H-Phe-Ala-His-OH (**1a**), a tripeptide acid containing His at the C-terminus. HATU (4 equiv.)/DIEA (8 equiv.)-mediated coupling of *o*,*p*-PALdehyde (4 equiv.) to an amino-functionalized resin containing an IRAA (H-Ile-PS, 1 equiv., 0.83 mmol/g) was carried out in DMF for 2 h, followed by on-resin reductive amination using HCl·H-Ala-OAllyl (10 equiv.) and $NaBH_3CN$ (10 equiv.) in DMF for 1 h.[6] Pd^0-promoted removal of the allyl ester was achieved as described in Scheme 1 [legend, part (i)]. Next, a solution of H-His(Trt)-O*t*Bu (10 equiv.) and DIEA (20 equiv.) in DMF was added to the amino acid-BAL-resin (1 equiv.), and the coupling (90 min) was initiated by addition of PyBOP[10] (10 equiv.) in solid form. Acylation of the secondary α-amino group was then performed, using the symmmetrical anhydride (5 equiv.) of Fmoc-Phe-OH in CH_2Cl_2–DMF (9:1) for 2+2 h [second coupling with fresh reagents, washes with DIEA–CH_2Cl_2 (1:19) before both couplings]. Final Fmoc removal, followed by acidolytic cleavage for 1 h with TFA–H_2O (9:1), provided the free

peptide (89% cleavage yield).[11] Analytical HPLC[12] of the crude material showed a single homogeneous component (t_R 12.5 min, >93% purity, condition A). Comparison to authentic standards of all possible diastereomers[13] led us to conclude that no racemization had occurred in any of the residues.[14,15] The same tripeptide was also prepared exactly as before, but starting with HCl·H-Ala-O*t*Bu instead of HCl·H-Ala-OAllyl. The *t*Bu group was removed using neat TFA, and the resin was washed with DIEA–CH_2Cl_2 (1:19) prior to coupling of H-His(Trt)-O*t*Bu. HPLC analysis of the final cleaved peptide indicated that no racemization had occurred (>94% final purity), and amino acid analysis on the peptide-resin[16] indicated that no premature loss of peptide occurred during the acid treatment that was used for on-resin *t*Bu removal.

Two hexapeptides containing a Pro residue at the C-terminus, H-Tyr-Gly-Gly-Phe-Ala-Pro-OH (**2**) and H-Tyr-Gly-Gly-Phe-Ala-Pro-N$(CH_3)_2$ (**3**), were prepared using the general protocol described for **1a** (allyl protection), except that HCl·H-Pro-O*t*Bu or H-Pro-N$(CH_3)_2$ were used at the appropriate steps. The final three residues were incorporated by DIPCDI/HOBt-mediated couplings in DMF for 1 h. After acidolytic cleavage for 1 h with TFA–H_2O (9:1), analytical HPLC[12] of the crude products showed a single component in both cases [for **2**, >95% purity, t_R 22.6 min (condition A); t_R 55.1 min (condition C); for **3**, >97% purity, t_R 22.7 min (condition A); t_R 57.9 min (condition C)].[17] Similarly, H-Tyr-Gly-Gly-Phe-Ala-Sar-N$(CH_3)_2$ (**4**) was prepared using the general protocol described for **1a** (*t*Bu protection), except that H-Sar-N$(CH_3)_2$ was used at the appropriate step. The final three residues were incorporated by DIPCDI/HOBt-mediated couplings in DMF for 1 h. After acidolytic cleavage for 1 h with TFA–Et_3SiH (9:1),[18] analytical HPLC[12] of the crude product showed a single component [>93% purity, t_R 21.7 min (condition A)].[19]

In conclusion, we have developed a new BAL approach for the N^{α}-Fmoc SPS of C-terminal modified peptides containing prolyl, *N*-alkylamino acyl, or histidyl derivatives at the C-terminus, which makes it possible to avoid diketopiperazine and racemization side reactions that plague alternative procedures. The key steps in the title approach involve manipulation of the resin-bound (penultimate) residue as the free amine, as opposed to the amide as in previous[6,7] BAL strategies. We believe that with the overall portfolio of three variations (original,[6] extended,[7] and new) of the BAL strategy, essentially any peptide with C-terminal modifications can be prepared in good yield with minimal side reactions, and that moreover, the approach can be readily extended for applications in the combinatorial library field.

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References

1. Abbreviations used: BAL, *B*ackbone *A*mide *L*inker, (Ref. 6), specifically as implemented in the tris(alkoxy)benzylamide system; DIPCDI, *N*,*N'*-diisopropylcarbodiimide; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt, 1-hydroxybenzotriazole; IRAA, 'internal reference' amino acid; NMM, *N*-methylmorpholine; *o*,*p*-PALdehyde, 4-[(4 or 2)-formyl-3,5-dimethoxyphenoxy]butyric acid; PyBOP, benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)-phosphonium hexafluorophosphate.

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8. The linkage between the BAL resin and the amino group is *completely stable* to the TFA concentration required to remove *t*Bu ester protection of the C^{α}-carboxyl group. For many cases, it is experimentally simpler, and allows for greater versatility with respect to availability of starting materials, to work with *t*Bu esters rather than allyl esters. Note that only *after* the amine is acylated does the linkage between the peptide and the resin become labile to high percentages of TFA.
9. We suggest that aminium/uronium coupling reagents should be avoided because of potential undesired guanidino formation between the coupling reagent and the BAL-anchored amine. See: Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *63*, 9678–9683.
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11. Amino acid composition of a hydrolyzed aliquot of peptide-resin: Phe, 0.80; Ala, 0.97; His, 0.96; Ile, 1.00. Hydrolysis after TFA cleavage: Phe, 0.09; Ala, 0.22; His, 0.22; Ile, 1.00.
12. Analytical HPLC was performed using either a Vydac C_{18} (0.46×25 cm) or a Vydac C_4 (0.46×15 cm) reversed-phase column, and linear gradients of 0.1% TFA in CH_3CN and 0.1% aqueous TFA were run at 1.0 mL/min flow rate from (condition A, Vydac C_{18}) 0:1 to 3:7 over 30 min; (condition B, Vydac C_4) from 0:1 to 1:49 over 30 min; (condition C, Vydac C_{18}) 8.5:91.5 to 11.5:88.5 over 60 min.
13. A reference sample of **1a** was prepared starting with *p*-alkoxybenzyl alcohol (Wang)-resin (0.8 mmol/g), to which the C-terminal Fmoc-His(Trt)-OH (10 equiv.) was esterified using DIPCDI (10 equiv.), and DMAP (1 equiv.) in DMF for 3 h. Further residues were incorporated by DIPCDI/HOBt-mediated couplings in DMF for 1 h. After acidolytic cleavage [TFA–H_2O (9:1), 1 h], HPLC analysis of the crude peptide showed two major peaks in a ratio of 7:2—the desired product and its racemic byproduct (H-L-Phe-L-Ala-D-His-OH, **1b**). (Compare to 15.4% D-formation reported in Ref. 4.) Standard samples of **1b**, H-L-Phe-D-Ala-L-His-OH (**1c**), and H-L-Phe-D-Ala-D-His-OH (**1d**) were synthesized by similar protocols. Good chromatographic resolution (Ref. 12) of all of the diastereomers was accomplished using conditions A: [(**1a**, t_R 12.5 min), (**1b**, t_R 11.5 min), (**1c+1d**, 14.3 min)] and B: [(**1c**, 14.5 min), (**1d**, 13.5 min)].
14. In a parallel experiment, **1a** was prepared as described in the main text but using a 5-min preactivation procedure for coupling of His. Amino acid composition: Phe, 0.19; Ala, 0.92; His, 0.33; Ile, 1.00. Analytical HPLC of the material revealed that no racemization had occurred (cf. Ref. 13).
15. Also, **1a** was prepared using the first generation BAL approach (Ref. 6), using H-His(Trt)-O*t*Bu (10 equiv.) and $NaBH_3CN$ (10 equiv). in HOAc–DMF (1:99) for on-resin reductive amination; remaining steps as in text. Amino acid composition of a hydrolyzed aliquot of peptide-resin: Phe, 0.93; Ala, 0.97; His, 1.00; Ile, 1.00. Analytical HPLC (Ref. 12) of the crude material revealed **1a**:**1b** = 93:7.
16. Amino acid composition of a hydrolyzed aliquot of peptide-resin: Phe, 0.82; Ala, 0.94; His, 0.93; Ile, 1.00.
17. Characterization: amino acid compositions of hydrolyzed aliquots of peptide-resins were: (**2**): Tyr, 0.77; Gly 1.58, Phe, 0.73; Ala, 0.99; Pro, 0.99; Ile, 1.00. (**3**): Tyr, 0.76; Gly 1.58, Phe, 0.72; Ala, 1.00; Pro, 0.99; Ile, 1.00. Cleavage yields after TFA–H_2O for 1 h were 95% (**2**) and 91% (**3**). MALDI-TOF MS, **2**: *m*/*z* calcd: 610.8, found: 611.2 $[M+H]^+$; **3**: *m*/*z* calcd: 637.8; found: 638.5 $[M+H]^+$, 660.5 $[M+Na]^+$.
18. Cleavage with TFA–H_2O (9:1) for 1 h resulted in 5–9% hydrolysis of the Ala-Sar bond. We also observed that TFA–H_2O cleavage results in hydrolysis of amino thioesters bound directly to BAL; this can be similarly prevented by using TFA–Et_3SiH. However, amino thioesters not bound directly to the BAL resin (see Ref. 7) do not hydrolyze.
19. Characterization: amino acid composition of a hydrolyzed aliquot of peptide-resin was: (**4**): Tyr, 0.78; Gly 1.62, Phe, 0.77; Ala, 0.94; Sar, n.d; Ile, 1.00. Cleavage yield after TFA–Et_3SiH for 1 h was 95%. MALDI-TOF MS, (**4**): *m*/*z* calcd: 611.8; found: 634.4 $[M+Na]^+$, 650.4 $[M+K]^+$.