Pergamon

Tetrahedron Letters, Vol. 38, No. 14, pp. 2531-2534, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)00393-6

Azido Acids in a Novel Method of Solid-Phase Peptide Synthesis.

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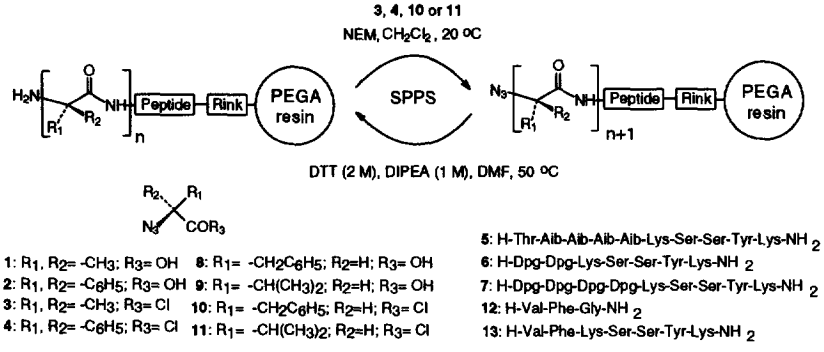
Abstract: Azido acids were produced from α-branched acids by α-bromination with NBS followed by substitution with sodium azide and the products were used in a novel method of solid-phase synthesis. The azido acids were transformed into the highly activated acid chlorides and used synthesis of extremely hindered peptides containing up to four successive diphenyl glycine or Aib residues. By reaction of the genetically encoded amino acids with TfN_3 and then $SOCl_2$ they were transformed into α-azido acid chlorides used in solid-phase peptide synthesis without racemization. © 1997 Elsevier Science Ltd.

The discovery of urethane protection for α-amino acids using the Cbz group led to the first essentially racemization free coupling reactions in peptide synthesis.[1] The carbonyl group of the urethane proved to have a sufficiently low nucleophilic character to prevent oxazolinone formation by reaction with the carbonyl carbon of the activated carboxyl group. The urethane protection was further developed into the acid labile Boc- and base labile Fmoc group[2,3] and these groups allowed the fast and quantitative deprotection of the α-amino function on solid-phase providing the ease of automation. The oxazolinone formation could also be suppressed with the *N*-Dts protecting group offering more complete protection of the nitrogen.[4] However, the *N*-Dts group is susceptible to nucleophilic attack and even weakly reducing conditions. Two classes of difficulties remain in peptide assembly, synthesis of aggregating peptides and of peptides involving sterically demanding acylation reactions. Aggregating peptides may now be synthesized by strategic use of backbone protection[5] or by introduction of pseudo-prolines.[6] The difficulties in sterically hindered couplings are more serious because the problems are inherent to the use of urethane protected amino acids which can form oxazolinones during demanding coupling reactions at the carboxyl group, activated to form a peptide bond.[7] This problem is particularly serious during coupling of α,α-dialkyl or diaryl glycines. Furthermore, sterically hindered amino nucleophiles may often show similar or less reactivity than the moisture present so strictly anhydrous conditions are required. Introduction of the small fluoride ion as leaving group using Fmoc amino acid fluorides as coupling reagents facilitated the synthesis of peptides containing several Aib-Aib bonds.[8]

In the present work a novel concept of a small size α-amino protecting group using azido acids was developed. The potential of azides as amino group precursors by reduction in solution has been demonstrated frequently in many fields of organic synthesis during the last 20 years,[9-15] however, many of the most convenient methods for quantitative reduction of azides are heterogeneous and not useful for solid-phase reactions. Recently, we described the use of DTT and other thiols for the efficient reduction of azido groups on carbohydrates bound to a solid phase.[13,16] Use of this quantitative reduction on α-azido acids has therefore allowed us to develop a novel method which uses the α-azido acids as versatile reagents for solid phase peptide synthesis, particularly of peptides subject to sterically demanding coupling reactions. Furthermore, the complete amine protection offered by the azide allowed high activation of the carboxyl group as acid chlorides in addition to conventional activation with *in situ* reagents or as fluorides or active esters.

2-Azido isobutyric acid **1** (Azib) was synthesized by bromination of isobutyric acid with NBS in refluxing CCl_4 for 3 days followed by substitution with NaN_3 in DMF (Figure 1). The product was obtained in 56% yield by silica gel chromatography. Similarly reaction sequence using diphenyl acetic acid and HPLC purification afforded 25% yield of 2-azido-2,2-diphenyl acetic acid **2** (Azdpa) together with impurities of diphenyl ketene and a small amount of 2-hydroxy-2,2-diphenyl acetic acid. The products were analyzed by IR, HPLC, elemental analysis, MS and NMR-spectroscopy.

The hindered α-azido acids were converted into the acid chlorides using an excess of thionyl chloride in CH_2Cl_2 at 50° C. The reaction was monitored by HPLC after quenching of the reaction with piperidine. It was found that Azib **1** and Azdpa **2** was converted completely to the acid chlorides **3** and **4** in <1 h and <2.5 h, respectively. These conditions were then used for successive conversion to acid chloride immediately before use. They could also be activated as acid fluorides using DAST as previously described[17] or by *in situ* activation with TBTU and NEM. However, the reactivities obtained with these methods were inferior to those of the acid chlorides.



First a PEGA resin was derivatized with 4-[Fmoc-amino-(2,4-dimethoxyphenyl)methyl]-phenoxyacetic acid (Rink linker) by activation with TBTU and NEM. A pentapeptide spacer facilitating MS-analysis, Lys-Ser-Ser-Tyr-Lys-NH- was assembled using Fmoc-amino acid-OPfp esters with Dhbt-OH catalysis and piperidine for Fmoc cleavage. Then the very sterically demanding peptides **5** and **6** were synthesized. The quantitative coupling of the acid chlorides required the addition of base to neutralize formed HCl. In the presence of NEM coupling of the first Azib-Cl **3** was complete in 30 min at 20° C. The reduction of the first α-azido group was performed with 2 M DTT and 1 M DIPEA in DMF at 50° C and was followed by MALDI-TOF-MS of the product cleaved off from a single resin bead. Under these conditions the reduction was rapidly progressing and was found to be quantitative within 30 min. The second coupling of **3** was quantitative within 40 min and the second reduction complete in 30 min. The third coupling was considerably more difficult and required 2 h to go to completion. The reduced rate reflect the fact of sterical interaction of the first Azib residue and the third coupling Azib residue in the extended peptide chain. The rate of the third reduction of azide with DTT was much decreased and 6 h was required for completion of the reaction. For the last coupling of **3** a coupling time 3 h was required to complete the reaction and the last azide was again reduced in 6 h. The coupling between Fmoc-Thr(tBu)-OPfp and Aib on the resin bound peptide was complete after 10 h. The yield of **5** was 75% crude, and 71% after HPLC purification. The product was analyzed by HPLC, MALDI-TOF-MS and proton NMR (Figure 2). The Azib was also activated with DAST to give the azido acid fluoride which coupled with the peptide resin, albeit at much less rate, and the reaction was complete in 6-8 h.

The coupling of Azpda-Cl **4** was significantly more difficult than that of Azib-Cl, indicating the large sterical hindrance imposed by the two geminal phenyl groups. The first coupling between Azdpa-Cl and Lys on the resin bound peptide was complete after 5 h, as followed by MALDI-TOF-MS. The reduction also was markedly affected by the increased sterical crowding of the two phenyl groups. The DTT reagent probably was too large and addition of a small amount of β-mercaptoethanol to the DTT/DIPEA reaction mixture enhanced

the rate of reduction which was complete within 24 h at 50° C. The coupling of the second Azdpa residue was complete in 24 h and the azide was quantitatively reduced with DTT/DIPEA/ME/DMF 24 h at 50° C. The yield of **6** was 60% after cleavage and purification by HPLC. Coupling of the third and fourth Azdpa residues was achieved in 36 h, while subsequent reductions required 40 h at 50° C. Prolonged reaction times were necessary and even acetylation with acetic anhydride required 5-10 h for completion.

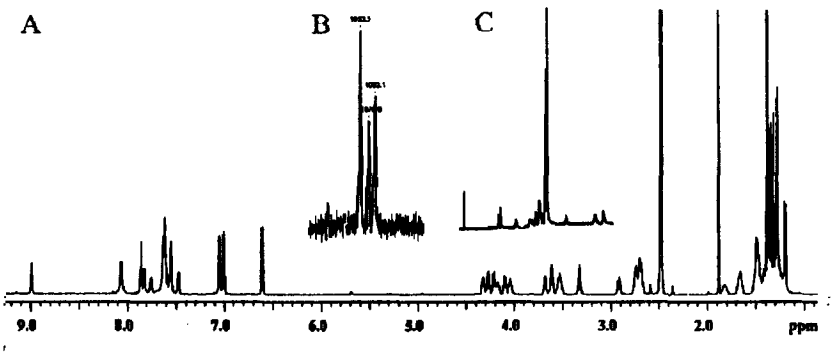


Figure 2. *^{1}H-NMR spectrum (A) and MALDI-TOF-MS (B, H^+, Na^+ and K^+ ions) of 5, synthesized by azido acid chloride couplings of Aib residues, and HPLC of the crude peptide (C).*

The genetically encoded free amino acids **8** and **9** could in high yield be converted into the azido acids **10** and **11**, with maintenance of the chirality of the α-carbon, by reaction with TfN_3.[10-12,18] The chiral stability and integrity was confirmed by measurement of optical rotation over a 3 day period in solution, and by high field NMR spectroscopy after peptide coupling of **10** and **11** reduction on the solid phase and cleavage from the resin. The neutral conditions of the diazo transfer reaction allowed the conversion of e. g. Phe-O*t*Bu to the azide in 90% yield (crude) without cleavage of the *t*Bu group. The method is currently being investigated for other acid labile side chain protecting groups frequently used in Fmoc based synthesis.

In order to study the use of the azido acid chlorides in standard peptide synthesis, the chiral azido acid analogs of Phe and Val were employed in the synthesis of **12** and **13** on the PEGA resin using a Rink linker (see above). The azido acid analogs of Phe and Val were activated as acid chlorides for 2 h with $SOCl_2$ at rt. The more difficult acylation reaction of Val to Phe to yield peptide **13** was monitored by MS, and was complete in <10 min. The reduction of Phe was quantitative in 2 h at 20 °C whereas the reduction of azide to give *N*-terminal Val was slower and required 1 h at 50 °C. Thus the reduction with DTT appeared to be sensitive to the steric hindrance imposed by the residue to which the azide is attached.

An analytical HPLC of the crude product of the short peptide **12** is shown in Figure 3. When D-Val-L-Phe-Gly-NH_2 was synthesized with TBTU activation and used as a reference compound, no racemization of crude **12** could be observed by HPLC or ^{1}H-NMR.[19] Purification by HPLC afforded **12** in 79% yield. The ^{1}H-NMR spectrum of the longer peptide **13**, after purification by HPLC (77% yield, Figure 3) was identical to the ^{1}H-NMR spectrum of **13**, synthesized using TBTU activation for coupling of Fmoc-Phe and Fmoc-Val. We currently study whether the more susceptible amino acids such as His and Cys are chirally stable under the conditions employed.

In conclusion, azides for protection of amino groups in solid phase synthesis offers an improved balance of steric requirements between the acylation reaction and the *N*-deprotection in the synthesis cycle. Furthermore, the complete protection observed with the azido function allows high activation of the carboxyl groups as acid chlorides. The general potential of this method for automated SPPS will be investigated.

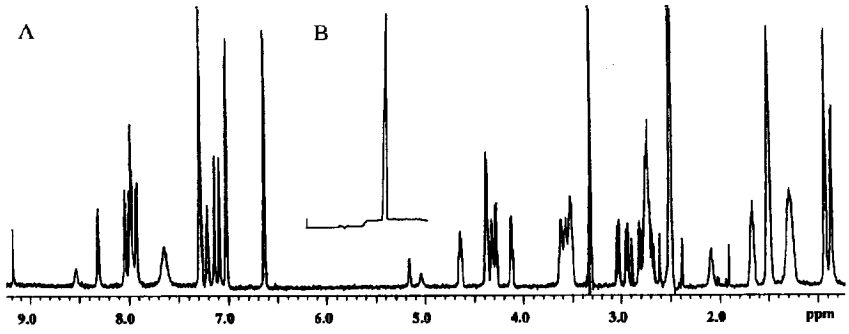


Figure 3. *^{1}H NMR spectrum of purified 13 (A) and HPLC of crude 12 (B) after cleavage from the resin both synthesized on solid phase by two successive azido acid chloride couplings and intermediate DTT reduction of the azide. No indication of racemization was observed*

Aknowledgement: This work has been supported by a travel grant (Maria A. Juliano) from FAPESP, Brasil

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18. **Hazard**: TfN_3 can detonate when handled in the purified form and should always be prepared and used *in situ* as a solution.
19. When performing the reduction at a more elevated temperature (55-60 °C, 1h), about 5% of racemization was observed for the short peptide **12**.

(*Received in UK* 3 *January* 1997; *accepted* 28 *February* 1997)