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A NOVEL DOUBLE DEPROTECTION-PEPTIDE CYCLISATION PROCEDURE AND ITS APPLICATION TO THE SYNTHESIS OF ANALOGUES OF THE CYCLIC TETRAPEPTIDE HC-TOXIN.

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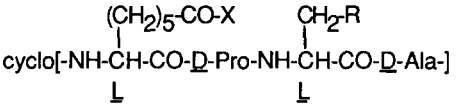
Summary: A hydroxide-mediated Fmoc/methyl ester double deprotection procedure followed by an improved bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl) cyclisation reaction are reported. This novel methodology was applied to the synthesis of three new HC-toxin analogues.

As an extension of our work[1] on the synthesis of analogues of the fungally derived, cytostatic and antimitogenic plant toxin, HC-Toxin,[2] cyclo[-Aoe-D-Pro-Ala-D-Ala-] (1),[3] we required analogues wherein [L-Ala][3] is replaced by L-iodophenylalanine (L-IPhe) and L-tyrosine. These compounds would then be amenable to the introduction of a radio-label in the form of ^{3}H or ^{125}I, and could be used as probes of the biological processes with which this agent interferes at the cellular level.

Our strategy was to utilise the methodology which we had previously developed[1] for the synthesis of [L-Phe][3]-HC-Toxin chloromethylketone (2), wherein the reactive epoxyketone moiety on the L-Aoe side-chain was replaced by a chloromethylketone resulting in biologically active synthetic analogues (IC_{50} for HC-Toxin (1), 10-15ng/mL; IC_{50} for HC-Toxin chloromethylketone (3), 30-40ng/mL).



1: X =  , R = H- (HC-Toxin)

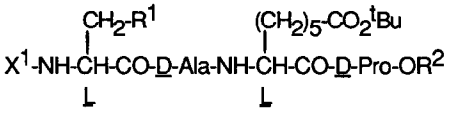
2: X = $-CH_2-Cl$, R = phenyl- ([L-Phe][3]-HC-Toxin chloromethylketone)

3: X = $-CH_2-Cl$, R = H- (HC-Toxin chloromethylketone)

In this approach, the protected linear tetrapeptide Z-Phe-D-Ala-Asu(O^tBu)-D-Pro-OMe (4)[4] was synthesised from the tripeptide $CH_3CO_2^-$ H_3^+N-D-Ala-Asu(O^tBu)-D-Pro-OMe (5) by using HOBt-catalysed active ester methodology.[5] Stepwise deprotection of the methyl ester followed by hydrogenolysis of the Z-protecting group liberated the cyclisation precursor, $CH_3CO_2^-$ H_3^+N-Phe-D-Ala-Asu(O^tBu)-D-Pro-OH (6), which was cyclised using the reagent bis(2-oxo-3-oxazolidinyl)phosphinic cloride (BOP-Cl)[6] in very dilute solution (CH_2Cl_2). This

strategy allows for the synthesis of a variety of analogues at position 3 and indicated that inclusion of L-IPhe or L-Tyr(OBzl) should be readily achievable. In these cases, however, the use of N-terminal Z-protection is precluded due to the hydrogenolysis-instability of L-IPhe and L-Tyr(OBzl). We thus required alternative amino-terminal protection.

A particularly attractive rationale involved use of the base-cleavable Fmoc-protecting group. By use of this functionality, we envisaged a double carboxy- and amino-terminal deprotection procedure whereby both the Fmoc-group and the methyl ester could be removed simultaneously under carefully controlled saponification conditions. This methodology would be particularly attractive if the crude product could then be cyclised directly.



7: X^1 = Fmoc-, R^1 = phenyl-, R^2 = -Me

8: X^1 = H-, R^1 = phenyl-, R^2 = -H

10: X^1 = Fmoc-, R^1 = 4-iodophenyl-, R^2 = -Me

11: X^1 = H-, R^1 = 4-iodophenyl-, R^2 = -H

13: X^1 = Fmoc-, R^1 = 4-benzyloxyphenyl-, R^2 = -Me

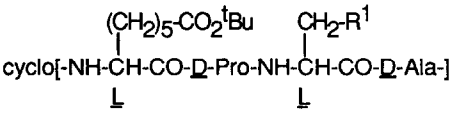
In a series of model reactions on Fmoc-Phe-D-Ala-Asu(O^tBu)-D-Pro-OMe (7)[7] (from 5 and Fmoc-Phe-OPfp;[8] HOBt, DIEA, DMF, r.t., 30-45 min), both the Fmoc-group and the methyl ester were removed in 6 hours on treatment with NaOH (3.3 equivalents) in aqueous methanol. Further study of this double deprotection indicated, firstly, a dependence of the reaction on solvent. Use of aqueous dioxane does not affect the expeditious removal of the Fmoc-group, as indicated by disappearance of starting material and rapid appearance of ninhydrin positive material on tlc, but the saponification of the methyl ester was still less than 50% complete after 52 h at room temperature with 4-5 equivalents of base. Under these conditions increasing amounts of ninhydrin positive by-products were also observed. These probably arise from epimerisation of one or more of the amino acids under the prolonged basic conditions. Secondly, the aqueous NaOH is best added in two portions. Both reaction time and byproduct formation appear to be reduced under these conditions.

On completion of the double deprotection (tlc), the reaction was worked up by extraction of the basic reaction mixture with ether to remove Fmoc-byproducts (usually dibenzofulvene and some 9-fluorenylmethanol formed by addition of OH^- to the dibenzofulvene), acidification to pH ≈3.5 with 1N HCl and finally, re-extraction with organic solvent. The water-soluble tetrapeptide, along with inorganic material, could then be obtained by lyophilisation. Rather than attempt to remove these inorganic salts, which consist mostly of NaCl, we attempted a direct cyclisation of the lyophilised powder. To our satisfaction, cyclisation of the crude H_2N-Phe-D-Ala-Asu(O^tBu)-D-Pro-OH (8) using BOP-Cl under high dilution (≤1 mM CH_2Cl_2) and in the presence of 3 equivalents of Et_3N and 2 equivalents of $NaHCO_3$,[9] led to isolation of a 24% yield of cyclic product (9). We have found that inclusion of $NaHCO_3$ and avoidance of acetate counter-ions in these reactions cause consistent and reproducibly higher yields of cyclic product.[10] It should also be noted that this cyclisation is highly heterogeneous; $NaHCO_3$ and the lyophilised powder are insoluble in CH_2Cl_2, although addition of Et_3N will cause the tetrapeptide to dissolve, and BOP-Cl dissolves slowly only on formation of the mixed carboxylic-phosphinic anhydride. The improved procedure described above would appear further to recommend BOP-Cl as a peptide cyclisation reagent.

Turning now to the iodophenylalanine-containing tetrapeptide, Fmoc-L-IPhe-OPfp {$[\alpha]_D$ -9.6° (c

1.5, $CHCl_3$); mp 178-181°C} was prepared by standard procedures (Fmoc-L-IPhe-OH,[11] C_6F_5OH, DCC, EtOAc, 0°C-r.t., 2 h;[8] 81%), and synthesis of Fmoc-IPhe-D-Ala-Asu(O^tBu)-D-Pro-OMe (10) from 5 was accomplished in 86% yield by using HOBt-catalysed active ester coupling[5b] (DMF, DIEA, r.t., 30-45 min). Double deprotection to yield 11, lyophilisation and cyclisation using BOP-Cl/Et_3N/$NaHCO_3$ yielded the product cyclo[-Asu(O^tBu)-D-Pro-IPhe-D-Ala-] (12) in 28% yield, providing further evidence for the utility of the double deprotection methodolgy and the improved cyclisation procedure.

Preparation of the Fmoc-L-Tyr(OBzl) tetrapeptide (13) was accomplished not by active ester methodology but by a recently described, racemisation-free acid chloride coupling procedure.[13] Thus, reaction of Fmoc-L-Tyr(OBzl)-Cl[13b] with 5 under the reported two-phase conditions (CH_2Cl_2, 10% aq. $NaHCO_3$, 30 min) proceeded smoothly to yield the desired tetrapeptide Fmoc-Tyr(OBzl)-D-Ala-Asu(O^tBu)-D-Pro-OMe (13) in 93% yield. As before, double deprotection and cyclisation yielded cyclo[-Asu(O^tBu)-D-Pro-Tyr(OBzl)-D-Ala-] (14) in 20% yield. In this case, the lower yield of cyclic product is probably related to the necessity for a more lengthy (9 h) saponification and consequent increase in byproduct formation. Hydrogenolysis of the -OBzl group (H_2, 10% Pd on C, MeOH) was then achieved in 88% yield to give cyclo[-Asu(O^tBu)-D-Pro-Tyr(OH)-D-Ala-] (15).



9: R^1 = phenyl-

12: R^1 = 4-iodophenyl-

14: R^1 = 4-benzyloxyphenyl-

15: R^1 = 4-hydroxyphenyl-

Thus we have demonstrated that by judicious choice of protecting groups, by the use of a novel Fmoc/methyl ester double deprotection procedure and an improved peptide cyclisation reaction, it is possible readily to synthesise analogues of the cyclic tetrapeptide HC-Toxin. The syntheses of the analogues described above also highlight the problems encountered with respect to efficient and controlled protecting group manipulation. The methodology we have developed circumvents these problems and should have applicability to other examples of highly functionalised and cyclic peptides.

Illustrative Procedures:

Double Deprotection of Fmoc-IPhe-D-Ala-Asu(O^tBu)-D-Pro-OMe (10):

To a solution of Fmoc-IPhe-D-Ala-Asu(O^tBu)-D-Pro-OMe (10; 0.35 g, 0.38 mmol) in MeOH (4 mL) was added 1 N NaOH (aq) in two portions (0.85 mL and 0.4 mL, 3.3 equivs. total) one hour apart. The reaction mixture was stirred at room temperature and the double deprotection was followed by tlc (silica, butanol/acetic acid/water, 4:1:1; Rf, H_2N-IPhe-D-Ala-Asu(O^tBu)-D-Pro-OMe, 0.65; Rf, H_2N-IPhe-D-Ala-Asu(O^tBu)-D-Pro-OH, 11, 0.54). After 5 h the reaction mixture was diluted with Et_2O (15 mL) and water (25 mL). The aqueous layer was was separated, re-extracted with Et_2O (2 x 15 mL) and acidified to pH 3-4 by addition of 1 N HCl (1.4 mL). The aqueous layer was extracted once more with Et_2O (15 mL) then lyophilised overnight to yield a white fluffy powder containing the product H_2N-IPhe-D-Ala-Asu(O^tBu)-D-Pro-OH (11; 0.38 mmol) and NaCl.

Cyclisation of H_2N-IPhe-D-Ala-Asu(O^tBu)-D-Pro-OH (11) using BOP-Cl:

The lyophilised powder containing H_2N-IPhe-D-Ala-Asu(O^tBu)-D-Pro-OH (11; 0.38 mmol) and NaCl was suspended in CH_2Cl_2 (400 mL) and a solution of Et_3N (0.13 g, 1.3 mmol) in CH_2Cl_2 (10 mL) was added. The resultant mixture was cooled to 0-5°C and covered with a blanket of N_2. Solid BOP-Cl (0.11 g, 0.44 mmol) was added followed immediately by solid $NaHCO_3$ (0.07 g, 0.8 mmol). The reaction mixture was stirred efficiently under N_2 and allowed to warm slowly to room temperature over 2-3 days. The solvent was evaporated and the residue was partitioned between EtOAc (40 mL) and brine (40 mL). The organic layer was separated and washed with saturated $KHSO_4$ (20 mL), saturated $NaHCO_3$ (20 mL), brine (20 mL), dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica (eluting solvent, EtOAc/hexane, 5:1) to yield the product, 12, (70 mg, 28%) as an oil.[7] Rf (silica, EtOAc/hexane, 10:1) 0.78.

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3) Abbreviations used in the text follow IUPAC-IUB rules as described in *Pure Appl. Chem.*, 1984, 56, 595. Additional abbreviations used: Aoe= L-2-amino-8-oxo-9(S),10-epoxydecanoic acid; Asu= L-2-aminosuberic acid; BOP-Cl= bis(2-oxo-3-oxazolidinyl)phosphinic chloride; DCC= N,N'-dicyclohexycarbodiimide; DIEA= N,N-diisopropylethylamine; Fmoc= fluoren-9-ylmethoxycarbonyl; HOBt= 1-hydroxybenzotriazole; L-IPhe= 4-iodo-L-phenylalanine; -OPfp= pentafluorophenyl ester.
4) Commercially available Z-L-Asu(O^tBu) is the precursor to the chloromethylketone-functionalised novel amino acid L-2-amino-8-oxo-9-chlorononanoic acid (L-Aoc).[1]
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10) BOP-Cl mediated cyclisation of 6 gave only a 7% yield of the product 9. Yields quoted are based on fully protected linear tetrapeptide.
11) Synthesised from L-iodoPhe using N-(fluoren-9-ylmethoxycarbonyloxy)succinimide (Fmoc-OSu) in dioxane/ aqueous Na_2CO_3 to avoid dipeptide impurities.[12] cf. Allen, M.C., Brundish, D.E., Wade, R., Sandberg, B.E.B., Hanley, M.R. and Iversen, L.L. (1982), *J. Med. Chem.*, 25, 1209.
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