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Synthesis of Fragments of Transforming Growth Factor Alpha Incorporating exo-2-Azabicyclo[2,2,1]heptane-3-carboxylic acids as Proline Substitutes

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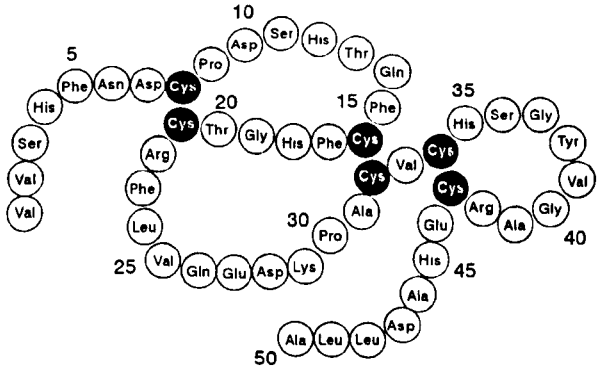
Abstract: *Two novel amino acids, the enantiomers of exo 2-azabicyclo[2,2,1]heptane-3-carboxylic acid have been independently synthesized by the aza Diels Alder reaction using a chiral auxiliary. The two acids have been advanced via solid phase synthesis to afford linear sequences, which have been cyclized to afford cyclic disulfide peptides, analogues of fragments of TGFα. The structures of these and other fragments have been firmly established.*

Cytokines and growth factors, which are involved in the regulation of cell division, growth and differentiation, are interesting targets for the development of new drugs.[1] In particular, the epidermal growth factor (EGF) receptor ligands, EGF and TGFα, are of great interest because of their role in normal physiological processes such as wound healing[2] and their involvement in the pathology of hyperproliferative[3] and neoplastic diseases[4]. EGF and TGFα have a characteristic motif containing three intramolecular disulfide linkages, giving rise to three loops.[5] Fragments of these growth factors[6] have been synthesized, but are of low biological activity. While this may reflect the complex interaction of the ligand with EGF-R,[7] which cannot be accommodated by use of a single linear sequence, it may also be due to failure of these peptides to adopt a conformation similar to that of the native ligand.



The primary structure of transforming growth factor alpha

The incorporation of un-natural α-amino acids into peptides represents a powerful approach to obtaining conformationally constrained oligopeptides. For example a conformationally constrained analogue of EGF_{33-42} has been synthesized in which glycine was replaced by 1-aminocyclopropane-1-carboxylic acid.[8] The present study details the synthesis of a TGFα fragment in which proline was replaced with a bicyclic analogue. The unique features[9] of proline (pyrrolidine structure and the N-alkylated amide bond) give rise to *cis* and *trans* rotamers of comparable energy. Hence the replacement of Pro-30 by novel un-natural proline analogues appeared to be an attractive strategy for study of structure activity relationships with peptides designed to interact with EGF receptors. Although there have been substantial efforts[10] to prepare proline analogues by variation of ring size, or by introduction of substituents at the 3, 4 and 5 positions, there are few details of their successful introduction into complex peptides. Here we report the enantioselective synthesis of bicyclic proline analogues and their incorporation by solid phase synthesis into peptide fragments based on $TGF\alpha_{21-32}$.

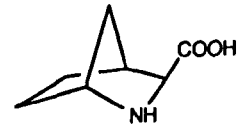
We have synthesized peptide (1) which corresponds to a modified fragment of TGFα, and three analogues of this peptide (2-4), (2) in which L-proline is replaced by D-proline and (3) and (4) in which L-proline is replaced by the enantiomers (5) and (6) respectively. The reasons for choosing the bicyclic analogues (5) and (6) were ease of synthesis and coupling (see below) and the belief that, additionally, the bicyclic skeleton might limit, close to this residue, the conformations available to the peptide.

Phe-Cys-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-Pro-Ala-Cys (1)

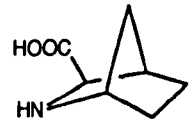
Phe-Cys-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-D-Pro-Ala-Cys (2)

Phe-Cys-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-(5)-Ala-Cys (3)

Phe-Cys-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-(6)-Ala-Cys (4)



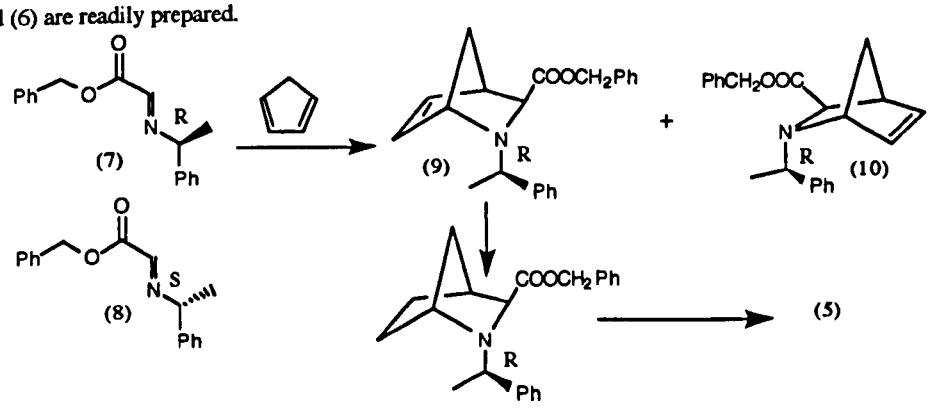
(5)



(6)

The synthesis of the amino acids (5) and (6) featured, as the key step, the aza Diels Alder reaction between cyclopentadiene and the chiral imines (7) and (8) derived from benzyl glyoxylate and (R)-α-methylbenzylamine and (S)-α-methylbenzylamine respectively. Although this particular Diels Alder reaction is novel related studies have been reported.[11,12] We chose the imines (7) and (8), so that later hydrogenolytic cleavage to afford the amino acids (5) and (6) would be easily achieved. Earlier use of chiral imines related to (7) and (8) have been limited to alkyl esters, which do not offer the same ease of isolation of the amino acids (5) and (6). However the earlier studies have been crucial in indicating the high level of diastereoselectivity which may be expected using these chiral auxiliaries, and by earlier X-ray studies[12] they permit absolute configurations of the final amino acids (5) and (6) to be readily assigned. Reaction of cyclopentadiene with imine (7) afforded two adducts (9) and (10) (ratio 3:1). The major isomer (9) had nmr features closely related to those of the exo-adducts earlier isolated and characterised by nmr[11,12] and by X-ray diffraction.[12] An exo structure could also be assigned to the minor isomer (10). Hence the Diels Alder reaction affords, in a manner similar to earlier studies, two exo adducts, which are readily separated by chromatography over silica gel. The substantial preference for formation of exo-adducts is noteworthy in view of our observation that an N-benzylimine, in contrast, affords both exo and endo adducts (58:42). The conversion of the major adduct (9), derived from imine (7), and of the

corresponding major adduct derived from the imine (8), to the required amino acids (5) and (6) respectively, was achieved in good yield [as shown for transformation of adduct (9) to amino acid (5)]. The enantiomeric purity of the amino acids (5) and (6) was checked (>95%) by chiral hplc of derivatives. Hence bicyclic proline analogues (5) and (6) are readily prepared.



Phe-Cys(R)-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-Pro-Ala-Cys(R) (11)

Phe-Cys(R)-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-D-Pro-Ala-Cys(R) (12)

Phe-Cys(R)-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-(5)-Ala-Cys(R) (13)

Phe-Cys(R)-Arg-Phe-Leu-Val-Gln-Glu-Asp-Lys-(6)-Ala-Cys(R) (14)

The synthesis of the peptides (1-4) was achieved by linear assembly via solid phase methods using fmoc chemistry followed by construction of the disulfide bond linking the cysteine residues. Assembly followed standard procedues using the following protecting groups, cysteine (trityl), arginine (pmc), aspartic and glutamic acids (t-butoxy) and lysine (boc). The peptide was cleaved from the HMP resin with 95% trifluoroacetic acid. An initial approach to the construction of the cyclic peptides was based on a linear synthesis with protection of the sulfhydryl groups of the cysteine residues by the S-acetamidomethyl group. Although removal of this protecting group by cyanogen iodide, silver salts or iodine has had some success, and alternatives such as the use of sulfoxides have been reported, we, in common with others,[8] find that such deprotections can be troublesome. Confronted with the failure to deprotect the linear peptides (11-14, R = S-Acm) we chose an alternative protection of the cysteine residues. Formation of the linear peptides (11-14, R = H) could be achieved by protection of the sulfhydryl groups with trityl groups. The trityl groups are then removed under the conditions of cleavage by TFA of the other protecting groups. The conversion of the linear peptides (11-14, R = H) to the cyclic peptides (1-4) was readily achieved by air oxidation. The conversion was essentially quantitative in ammonium bicarbonate solution at 38°C. In order to ensure the satisfactory purity of the cyclic peptides a final purification by hplc was achieved by gradient elution. The authenticity of the peptides was established by a variety of criteria. All were homogeneous by hplc. Mass spectrometric results established the required molecular weights and amino acid analysis showed the presence of all residues other than the unnatural amino acids and cysteine. Detailed 2-D nmr results, to be published elsewhere, enabled the amino acid sequences to be confirmed. Hence these results establish the satisfactory synthesis of the TGFα fragment (1) and the analogues (2-4). In particular they show how the bicyclic proline analogues (5) and (6) can be successfully incorporated into peptides. The importance of this achievement is underscored by our difficulty in achieving a similar incorporation of 2-methylproline into peptides. In this latter case the degree of extra steric

hindrance interferes with the coupling methodology. In contrast coupling with the amino acids (5) and (6) presents no significant problems. The full details of studies concerned with the observed biological activity associated with the cyclic peptides (3) and (4), the lower activity of the cyclic peptides (1) and (2) and the inactivity of the linear peptides (11-14), and the conformational characteristics of the peptides (1-4), will be reported elsewhere. However CD studies establish the similar conformation of peptides (1) and (3), and again of peptides (2) and (4). Therefore the bicyclic amino acids (5) and (6) behave as good analogues of D-proline and L-proline, and should permit an elucidation of the subtle role associated with proline residues, where the importance of cis peptide bonds can control peptide conformation. The first preparation of the enantiomer (6) by a different Diels Alder approach has been recently published[13] and emphasizes the interest in the incorporation of constrained proline analogues in the place of proline into biologically active peptides.

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