Pergamon

Tetrahedron Letters 43 (2002) 3057–3059

TETRAHEDRON LETTERS

Optimised solid phase synthesis of a cystine-linked peptide-PNA chimera

Enrico Millo,[a,†] Raffaele Nicolai,[a,†] Sonia Scarfi',[a] Carlo Scapolla,[a] Barbara Biasotti,[b] Umberto Benatti[a] and Gianluca Damonte[a,*]

[a]*Department of Experimental Medicine, Section Biochemistry, University of Genoa, Viale Benedetto XV, 1, 16132 Genoa, Italy*
[b]*Laboratory of Experimental Oncology, National Institute for Cancer Research, 16132 Genoa, Italy*

Received 15 February 2002; accepted 21 February 2002

Abstract—A solid phase synthesis method for a cystine-linked peptide-PNA chimera starting from different modified protected cystine monomers was developed. This strategy dramatically improved the final yield and the specificity of disulphide bond formation in this kind of oligomers, which are traditionally obtained by liquid phase coupling via oxidation of the two terminal cysteines.

Peptide nucleic acids (PNA)[1,2] are DNA analogues with powerful resistance to nucleases and proteases in the cellular environment while retaining the base pairing properties.[3] Accordingly, they could be powerful tools for gene expression tuning. A strong limit of PNAs is their limited permeability to cellular membranes,[4] prompting researchers to perform couplings to hydrophobic molecules such as dihydrotestosterone,[5] or to signal peptides able to cross the membranes.[6] This raises the question of whether such moieties could hinder or modify PNA function.

We synthesised a signal peptide-PNA chimera with the two moieties linked by a disulphide bridge involving two cysteines, a very redox-sensitive bond susceptible of being rapidly cleaved once inside the cell by the reducing cytoplasmic environment. Traditionally, these heterodimers are obtained by synthesising the PNA and the peptide, each bearing a terminal cysteine, and subsequently oxidising the thiols to form a disulphide bridge in liquid phase using sulphydryl-reactive groups such as maleimide, dithiopyridyl and bromoacetyl.[7] This strategy results in very low yields and does not guarantee specificity in the formation of the disulphide bridge in the presence of other possible reactive groups (e.g. amino, carboxyl and hydroxyl).

Performing the synthesis of such a molecule, we aimed at minimisation of two problems: one is the coupling of a single cystine monomer (with two carboxyl groups) to two growing peptide chains (a double coupling), which effectively results in termination of chain growth; the second is disulphide bond breakage during the final cleavage, a frequent occurrence we observed in preliminary experiments. To avoid the "double coupling" we tested three different strategies which are summarised in Fig. 1. The sequence synthesised was T-(N-linker-Cystine)-GKS by a solid phase standard Fmoc protocol. Also, we used a fourth strategy synthesising the sequence ctt-(N-linker-Cystine)-VKRKKKP (lower cases indicate PNA bases and the peptide portion corresponds to the SV40 Nuclear Localisation Signal[8]).

In strategy A (Fig. 1A), the coupling of the *t*-Boc-protected cystine with two free carboxyl groups (Nova Biochem AG, Laufelfingen, Switzerland) at the amino groups of the growing peptide chain was followed by the coupling of an N-linker (mono-Fmoc ethylenediamine hydrochloride) (NovaBiochem) in the solid phase. The N-linker was used to re-form the N-terminal functional group. In strategy B (Fig. 1B), the cystine was previously protected at only one carboxyl-end using 2-chlorotrityl chloride[9] (Nova Biochem). In strategy C (Fig. 1C) the cystine and the N-linker were previously coupled in liquid phase synthesis and the resulting dimer coupled to the growing chain. Finally,

Abbreviations: amino acids and peptides follow the IUPAC-IUB nomenclature; PNA, peptide nucleic acid; Fmoc, 9-fluorenylmethoxycarbonyl; *t*-Boc, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid; DMF, *N*,*N*-dimethylformamide; NMP, *N*-methyl-2-pyrrolidone; DCM, dichloromethane.

Keywords: PNA; disulphide bond; SPPS.

* Corresponding author. Tel./fax: 39-010-5737270; e-mail: damonte@cba.unige.it

† The two authors contributed equally to this work.

0040-4039/02/$ - see front matter
PII: S0040-4039(02)00373-8

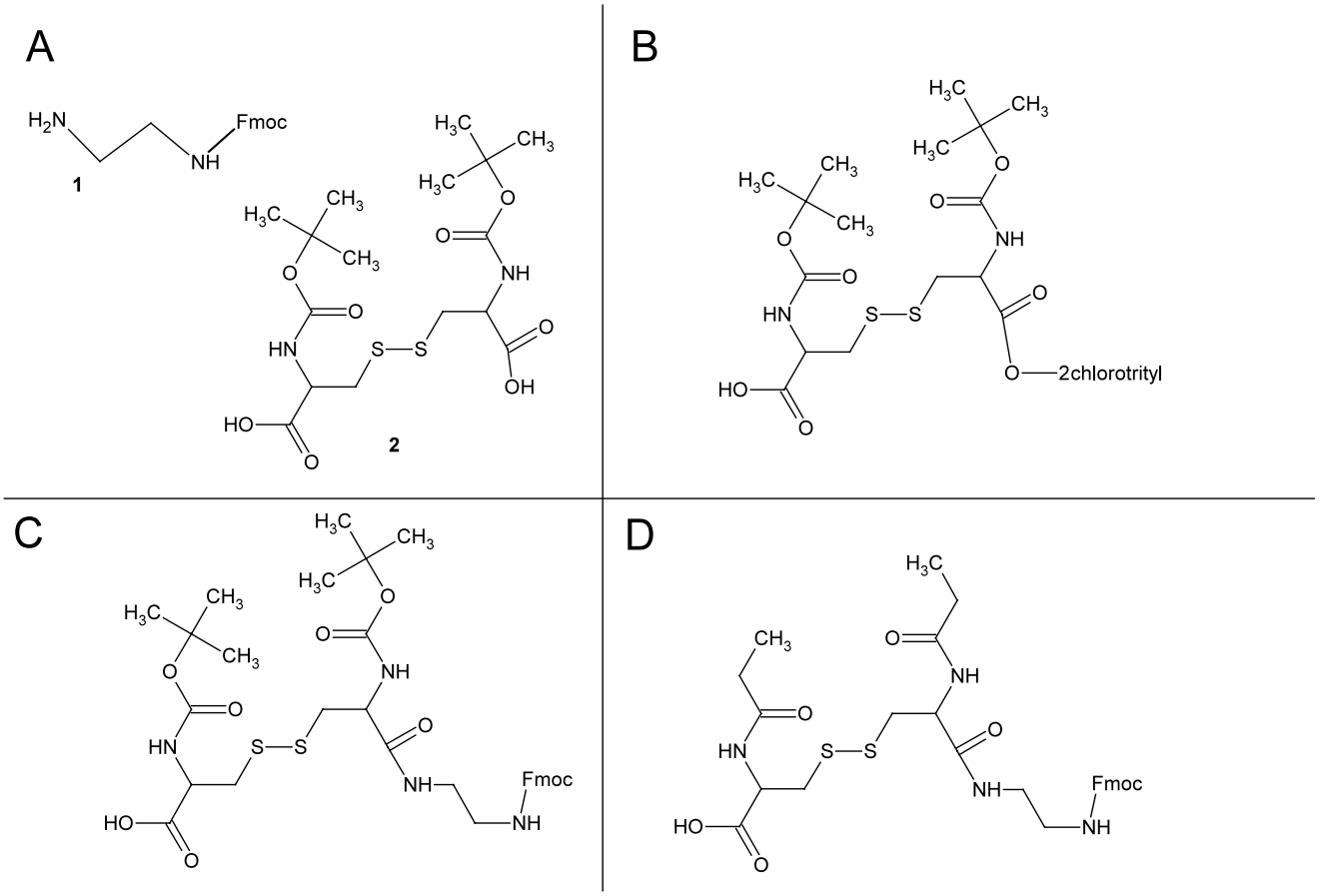

Figure 1. The four strategies employed for the construction of the chimera. (A) N-linker (**1**) and cystine (**2**) as commercially available. (B) The monoprotected 2-chlorotrytil-cystine. (C) The cystine-N-linker dimer obtained by liquid phase synthesis. (D) The previous dimer permanently protected on the amino groups by propionylation.

in strategy D (Fig. 1D), the N-linker-cystine dimer was modified further by cleaving the protecting *t*-Boc groups on the cystine and introducing in their place two TFA-uncleavable permanent protections by propionylation.

All peptides were synthesised using a standard solid phase Fmoc protocol,[10] in particular coupling was carried out for 40 min with 4 equiv. of amino acid, 3.8 equiv. of activator [*O*-(7-azabenzotriazol-1-yl-)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU), 4 equiv. of sim-collidine and 6 equiv. of *N*,*N*-diisopropylethylamine (DIPEA) in NMP at 40°C. In synthesis A (strategy A), we used a reduced amount (2 equiv.) of HATU for the cystine coupling, to prevent the simultaneous activation of both carboxyl groups, while for the N-linker coupling we pre-activated the carboxyl groups on the resin to avoid reaction between the HATU and the free amino group of the N-linker.[11] For strategy B the synthesis of the monomer was performed as previously described.[9] The purity of the products was checked with an electrospray single quadrupole mass spectrometer (ES-MS) in negative ion mode giving a 90% final yield. After coupling the monomer to the growing peptide chain, the protection was removed by 1% TFA in DCM for 1 minute, followed by a 5% pyridine in DMF wash. For synthesis C the cystine-linker dimer was obtained by slowly and continuously dropping the activator (HATU) in DCM:DMF (1:1) to a mixture of the two monomers and DIPEA (each component at 50 mM). The dimer was then purified by silica gel chromatography (DCM:CH_3OH 20:1) and its purity, more than 95%, checked by ES-MS in negative mode. In strategy D we proceeded as in strategy C adding two steps: the protecting *t*-Boc groups were removed by treatment with TFA and, subsequently, the amino groups were acylated by propionic anhydride. The reaction was also monitored by ES-MS, in negative ion mode, with an almost 100% final yield.

We performed an ES-MS analysis of each chimera and observed very poor yields for strategies B and D. In strategy B probably the disulphide bond was cleaved by the TFA during the trityl removal step (see below). In synthesis D, we hypothesise that the two propionyl groups could hamper the activation of the carboxyl by the HATU; this is supported by the presence, observed by ES-MS analysis, of the uncoupled PKKKRKV peptide, meaning that our dimer did not react with the growing chain.

Although in synthesis A we adjusted the concentration of the activator, the final yield of the PNA-peptide was below 20%. The best yield of the chimera, over 60%, was obtained with strategy C.

However, frequently an important fraction of the synthesis did not correspond to the chimera, but to another molecule: a 'bent' palindromic peptide or PNA (VKRKKKP-Cystine-PKKKRKV or ctt-Cystine-ttc), that is, the peptide or PNA dimers formed by the cystine linked to two growing chains (Fig. 2). We observed these 'bent palindromic' forms when the syntheses were begun both with PNA and with peptide moiety. The phenomenon was quite predictable in strategy A, but we were surprised to observe the same side reaction also when the Cystine-N-linker dimer was used (strategy C). Whereas in the former case we can certainly indicate the 'double coupling' as the cause of the palindromic sequence formation, in strategy C we could hypothesise that the strong acidic environment during the cleavage step causes a radical-mediated exchange reaction involving the free orbital of the sulphur;[12] to test it, we monitored the chimera:palindromic sequence ratio variation during a time course cleavage experiment where we observed that a 2 hour cleavage leads to 55:45 ratio between the ctt-(N-linker-Cystine-)-PKKKRKV and the palindromic dimer VKRKKKP-(Cystine)-PKKKRKV, while a 24 hour reaction results in a 35:75 ratio of the same molecular species, indicating this as a crucial point for the disulphide breaking and dimer exchange.

Nonetheless, we have now developed a reasonably efficacious protocol for the synthesis of a molecule for use in cell delivery experiments, with the expectation that the sensitive disulphide bond will be quickly cleaved intracellularly. Our next goal is to further optimise the synthesis and to study the cellular stability of the chimera. We already performed preliminary experiments on cytoplasmic disulphide bridge reduction which show a rapid separation of the two moieties once the chimera has entered the cells (see Table 1). In fact,

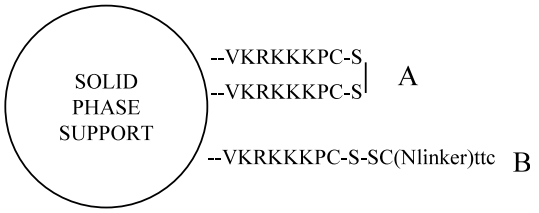

Figure 2. The two products obtained during our synthesis. (A) The undesired 'bent palindromic' peptide. (B) The desired chimeric sequence.

Table 1. The intracellular fate of the chimera ctt-(N-linker-Cystine)-PKKKRKV: numbers are expressed in nmole/mL of cellular extracts

Time	Chimera	Cys-N-linker-PNA	N-linker-PNA
1 min	0.758	0.118	0.037
15 min	0.836	0.037	0.033
30 min	0.736	0.032	0.018
60 min	0.590	0.050	0.045
2 h	0.570	0.110	0.092
6 h	0.405	0.370	0.513
24 h	0.064	0.075	1.376

HPLC-ESI-MS analyses of cellular extracts of 1×10^6 HeLa cells treated with 100 μM of the chimera in culture medium in a time course experiment clearly showed that internalisation of the chimera leads to the time-dependent formation of two molecular species: the first is the Cys-N-Linker-PNA, generated by the disulphide bridge reduction, while the second, derived from the former and lacking the C-terminal cysteine, is due to enzymatic cleavage of the cysteine itself by a non-specific carboxypeptidase activity. Nonetheless, both molecular species could be able now to exert their inhibitory effect on DNA or mRNA complementary target sequences.

Acknowledgements

This work was supported by CNR funds on Target Project 'Biotecnologie' and on Strategic Project 'Drug Delivery'. We thank Dr. Roberto Stock Silberman for critical reading of the manuscript.

References

1. Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497–1500.
2. Egholm, M.; Buchardt, O.; Nielsen, P. E.; Berg, O. *J. Am. Chem. Soc.* **1992**, *114*, 1895–1898.
3. Demidov, V. V.; Potaman, V. N.; Frank-Kamenetskii, M. D.; Egholm, M.; Buchardt, O.; Sonnichsen, S. H.; et al. *Biochem. Pharmacol.* **1994**, *48*, 1310–1313.
4. Wittung, P.; Kajanus, J.; Edwards, K.; Haaima, G.; Nielsen, P. E.; Norden, B.; Malmstrom, B. G. *FEBS Lett.* **1995**, *375*, 27–29.
5. Boffa, L. C.; Scarfi', S.; Mariani, M. R.; Damonte, G.; Allfrey, V. G.; Benatti, U.; Morris, P. L. *Cancer Res.* **2000**, *60*, 2258–2262.
6. Pooga, M.; Soomets, U.; Hallbrink, M.; Valkna, A.; Külliki, S.; Rezaei, K.; Kahl, U.; Hao, J.; Xu, X.; Wiesenfeld-Hallin, Z.; Hökfelt, T.; Bartfai, T.; Langel, Ü. *Nat. Biotechnol.* **1998**, *16*, 857–861.
7. Drijfhout, J. W.; Hoogerhout, P. In *Methods of Preparing Peptide-Carrier Conjugates*; Chan, W. C.; White, P. D., Eds. Fmoc solid phase peptide synthesis: a practical approach. Oxford University Press: New York, 2000; pp. 231–236.
8. Brandén, L. J.; Mohamed, A. J.; Smith, C. I. E. *Nat. Biotechnol.* **1999**, *17*, 784–787.
9. Athanassopoulos, P.; Barlos, K.; Gatos, D.; Hatzi, O.; Tzavara, C. *Tetrahedron Lett.* **1995**, *36*, 5645–5648.
10. Casale, R.; Jensen, I. S.; Egholm, M. In *Synthesis of PNA Oligomers by Fmoc Chemistry*; Nielsen, P. E.; Egholm, M., Eds. Peptide nucleic acids protocols and applications. Horizon Scientific Press: Norfolk, UK, 1999; pp. 39–50.
11. Schnoelzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Peptide Protein Res.* **1992**, *40*, 180–193.
12. Mezyk, P. S.; Armstrong, D. A. *J. Chem. Soc., Perkin Trans. 2* **1999**, 1411–1419.