

**METHOD FOR PRODUCING INTERFERON**Field of the invention

The present invention relates generally to methods of producing interferon proteins comprising one or more cysteine residues and two or more polypeptide subunits, by chemical  
5 synthesis. The invention further relates to subunits constituting the interferon proteins and to their use in the production of biologically active interferon.

Background of the Invention

10 Interferons are single-chain proteins released by cells invaded by viruses or certain other substances. Interferons are presently grouped into three major classes, designated leukocyte interferon (interferon-alpha, alpha-interferon, IFN-alpha, molecular weight 19kDa, approximately  
15 165 amino acids), fibroblast interferon (interferon-beta, beta-interferon, IFN-beta, molecular weight 19KDa, approximately 165 residues), and immune interferon (interferon-gamma, gamma-interferon, IFN-gamma, molecular weight 17kDa, approximately 140 residues).

20 The known IFN-alphas contain two disulfide bonds, the IFN-betas contain one disulfide bond, and IFN-gammas have no disulfides. These disulfide bonds are formed by oxidation of the side-chains of cysteine residues. For example, the amino acid sequence of mature recombinant IFN-alpha A contains  
25 cysteine residues at positions 1, 29, 98 and 138.

Intramolecular disulfide bonds are formed between the cysteine residues at positions 1 and 98, and between the cysteine residues at positions 29 and 138.

Interferons have a variety of biological activities,  
30 including antiviral, immunoregulatory and antiproliferative properties, and are, therefore, of great interest as

therapeutic agents in the control of cancer and various viral diseases, notably Hepatitis C and Hepatitis B. Thus, there exists a need for methods that provide for an efficient production of interferons for use as a therapeutic.

5           Current commercial methods for the production of interferon involve the synthesis of recombinant proteins in bacterial or mammalian cells. For example, interferon-alpha is produced by harvesting bacteria containing a plasmid encoded with the gene for the interferon-alpha protein, e.g.  
10 described in US-5,710,027, US-6,005,075, and Hochuli, *Chimia* 40, 408 (1986). The bacteria containing the interferon plasmid is then induced to synthesize interferon-alpha, and at the appropriate time the cells are lysed and the contents of the cell collected. The interferon-alpha is present in  
15 reduced form and contaminated with many other proteins. This crude mixture is subjected to conditions to oxidize the cysteine residues to form the two specific disulfide bonds essential for biological activity. This mixture of native interferon, mis-folded interferon and the other contents of  
20 the cell lysate are then subjected to a series of purification techniques to obtain purified interferon. Nevertheless, the bacteria that produces the desired interferon protein are littered with proteins of similar sizes and charge distributions, and these contaminating  
25 proteins make the purification process expensive and sometimes impossible to perform without either an unacceptable loss of the desired interferon protein or presence of contaminating proteins.

          Staehelin et al., *J. Biol. Chem.*, 256, 9750-9754  
30 (1981) described the preparation of recombinant interferon-alpha from *E. coli* and the purification of the cell lysates using immunoaffinity column chromatography where the column resin is derivatized with a specific antibody that binds the

interferon-alpha. While the immunoaffinity column used in the Staehelin method selectively binds the interferon-alpha, the cost of immunoaffinity columns is high and the useful life of the column is limited.

5           A particularly difficult contaminant from the recombinant production of interferon-alpha is the interferon-alpha product comprising an added methionine at the N-terminus. This contaminant is difficult to remove during purification because it is so similar to interferon-alpha in  
10 charge, size and tertiary structure. To overcome this problem Hochuli (1986) was only able to remove this contaminant by using immunoaffinity chromatography and three additional purification steps. In addition, the use of immunoaffinity columns to purify the recombinant interferon-alpha introduces  
15 the risk of contamination by viruses or by murine immunoglobulin in the final product.

To avoid the expense and aforementioned problems associated with immunoaffinity chromatography it was found necessary in US-5,710,027 and Ettlin et al., (1999) to use  
20 multi-step purification procedures (four different methods in each case).

It has also been recognized in US-5,935,566 that recombinant interferon-alpha might be unstable during storage. For instance, one component of their formulation,  
25 EDTA (ethylene diamine tetra acetate) was found to prevent the formation of visible particulate matter in solutions of interferons. This beneficial effect of EDTA is presumed to result from its scavenging of trace metal cations such as  $\text{Cu}^{2+}$ , which is used in metal chelate chromatography to purify  
30 interferon proteins (Ettlin et al., 1998). Thus, it is evident that many problems still exist in the preparation of highly pure interferon from recombinant proteins.

Various methods for the chemical synthesis of polypeptides are described in the art. The most common method for the preparation of peptides is by Solid Phase Peptide Synthesis (SPPS). In this method the peptide chain is built  
5 by sequential addition of amino acids to a macroscopic, insoluble support. Addition of each amino acid requires two reactive steps, the deprotection of the N-terminus of the resin-bound peptide and acylation of the resultant free amino group by the next, N-protected, C-alpha activated amino acid.  
10 After each step excess reagents and byproducts may be removed simply by washing the polymeric resin. Once the desired sequence of side-chain protected amino acids has been assembled the peptide is cleaved from the resin with concomitant removal of the side-chain protecting groups and  
15 purified by reversed-phase HPLC (RP-HPLC) or other methods which are known in the art. Different methods of applying this general principle are known. The two most common are the Boc (t-butyloxycarbonyl, Kent, 1988) and Fmoc (9-fluorenyloxycarbonyl, Fields and Noble, 1990) techniques,  
20 named after the groups used to protect the N-alpha moiety of the C-activated amino acids.

The above process is usually efficient for the synthesis of short to medium length peptides. However even with the excesses of reagents commonly employed, reaction  
25 yields are less than 100% and the unreacted species accumulate on the resin. For example, to synthesize a peptide of 50 residues requires 100 reactive steps of very high yield. Even if the efficiency of each step was 99% then effectively none of the target peptide would be obtained. In  
30 addition the final cleavage step which also removes the side-chain protecting groups can generate species which can modify the target peptide. Following the cleavage of the polypeptide from the resin with concurrent removal of the side-chain

protecting groups the desired peptide is initially obtained contaminated by shorter peptides (deletion or truncation sequences), some of which have physicochemical properties (size, charge, hydrophobicity) very similar to that of the target peptide and from which it can be very difficult to obtain the desired peptide in pure form. Thus, as the polypeptide sequence becomes longer, it tends to be increasingly difficult to both synthesize and purify the desired sequence to obtain it in practical quantities. It is generally accepted that the synthesis of polypeptides longer than about 50 amino acids, though possible, is far from routine (see e.g. Kochendoerfer and Kent, *Curr. Opin. Chem. Biol.* 3, 665-671 (1999), Brik et al., *J. Org. Chem.* 65, 3829-3835 (2000)). These inefficiencies make stepwise synthesis an impractical commercial approach to the production of large proteins, such as interferon proteins.

In order to overcome these limits to the length of peptides producible by SPPS, complementary techniques have been developed. Of interest to the present invention are processes by which fragments of a protein may be synthesized using solid phase synthesis and then combined to form the full length protein. Thus, Dawson et al., *Science*, 266: 776-779 (1994) and US-6,184,344 describe the use of native chemical ligation to combine two sections of a larger protein to form a full length protein. By this method, polypeptides are prepared in a range of lengths, including 11, 39, 72, 99 and 110 amino acids long. The native chemical ligation reaction is carried out to ligate a first peptide, of which the N-terminal residue is a Cys with a reduced sulfhydryl moiety, with a second peptide, in which the alpha-carboxylate has been converted to a thioester. In aqueous solution around neutral pH the peptide-C-alpha-thioester can react chemoselectively with the N-terminal Cys on the second

peptide to form a side-chain thioester-linked intermediate. This spontaneously undergoes intermolecular rearrangement to form a native peptide bond at the ligation site, and to regenerate the sulfhydryl group of the Cys side-chain. An  
5 advantage of this technique is that the peptide fragments are fully deprotected and thus are highly soluble in the aqueous solvents used to purify them and in the ligation step. Nevertheless, Kent et al. (1995) only teach the ligation method to form proteins as large as 110 amino acids and not  
10 larger proteins such as human interferon-alpha, comprising 165 amino acid residues.

A limitation of native chemical ligation was the requirement for a Cys at the N-terminal of the C-terminal peptide fragment. To overcome this limitation and to allow  
15 ligation between other residues auxiliary groups have been disclosed. These bear  $\beta$ -amino thiols, e.g. Low et al., Proc. Natl. Acad. Sci. USA, 98, 6554, (2001), Kawakami et al. Org. Lett. 3: 1403 (2001) or  $\gamma$ -amino thiols, e.g. Canne et al., J. Am. Chem. Soc. 118, 5891 (1996). In order to regenerate the  
20 native residue at the ligation site the auxiliary comprises a moiety allowing cleavage of the auxiliary from the peptide, e.g. by strong acid.

Since the disclosure of the technique of native chemical ligation, it has been used frequently to make  
25 proteins. The polypeptides prepared by this technique are typically 70-110 residues long (e.g. SDFaN33A (68 residues), Ueda et al., J. Biol. Chem. 272, 24966 (1997), Eglin C (70 residues), W.-Y. Lu et al., Biochem. 39, 3575 (2000) and barnase (110 residues), US-6,184,344). The longest  
30 polypeptide that has been demonstrated to have been prepared in high purity by native chemical ligation is human type II secretory phospholipase A2 (124 residues) (Hackeng et al. Proc. Natl. Acad. Sci. USA, 94, 845 (1997)).

In order to produce even longer proteins sequential native chemical ligation has been developed (Hackeng et al., Proc. Natl. Acad. Sci. USA, 96: 10068 (1999)). This technique uses the same reaction as native chemical ligation, but three or more fragments are sequentially ligated together. The middle fragments comprising both a C-alpha thioester and an N-terminal Cys residue must have a protecting group on the side chain or N-alpha of the Cys to prevent the peptide reacting with itself to form a cyclic peptide or with other molecules of the same sequence to form oligomers. Initially the protecting group used was the acetomidomethyl group, Acm (Veber et al., J. Am. Chem. Soc. 94: 5456 (1972)) or the methylsulfonyl ethyloxycarbonyl (Tesser et al., Int. J. Peptide Protein Res., 7 : 295 (1975)). Removal of the Acm group requires treatment of peptides with mercury salts, often under strongly acidic conditions, and subsequently with  $\beta$ -mercaptoethanol. These conditions risk modification of the polypeptide, e.g. Asp-Gly sequences may be sensitive to strong acid, which can cleave the peptide backbone. Careful purification is required to ensure that all mercury is removed from the polypeptide. The Msc group is removed under strongly basic conditions which can also modify polypeptides, e.g. dehydration of Asn residues.

On completion of the first native ligation reaction the mixture is purified, the N-terminal Cys of the ligated peptide is then deprotected and the mixture is purified. The product of the first ligation then takes part in another native chemical ligation with a third peptide which has a thioester group. This process is repeated to assemble the entire sequence of the polypeptide. This, in theory, allows even longer polypeptides to be prepared in high purity. However the examples that have been disclosed are of similar length as those produced by native ligation of two fragments,

e.g. three-step, four-fragment synthesis of human secretory phospholipase A2 (124 residues) (Hackeng et al. (1999)).

A disadvantage of the sequential ligation technique is that the additional purification steps necessary make this  
5 time-consuming, labor-intensive and therefore costly as a method of production. Methods to accelerate this sequential ligation by assembling the ligated fragments on a polymeric resin have been disclosed by Canne et al., 1999, who synthesized the 118 residue human group V secretory  
10 phospholipase A2 by this method. A disadvantage of this "Solid-Phase Protein Ligation" approach is that non-standard linkers must be prepared and used to add the purified C-terminal fragment to the resin on which the fragments are assembled by native ligation.

15 Native ligation relies on two key steps. One is the ligation step to join the two peptides. The second is the preparation of a peptide-alpha-thioester. In the method disclosed by Dawson et al. (1994) the peptide thioester was synthesized on a resin with a linker that, on cleavage of the  
20 peptide, yielded a peptide thioacid. This thioacid group was subsequently transformed into a thioester in solution. Linkers have since been disclosed that afford peptide-alpha-thioesters directly on cleavage (e.g. Hackeng et al. (1999)). These linkers are only useful if the stepwise assembly of the  
25 peptides is by the Boc method. Although useful for the rapid synthesis of medium length peptides, the Boc method is less efficient in the synthesis of longer peptides. In fact it has been noted that for native chemical ligation, the process requires use of fairly pure peptides, limiting the length of  
30 precursor fragments employed in each ligation step to 30-35 residues (see Villain et al. (2001)). In addition, to obtain peptides in reasonable purity by Boc chemistry the cleavage

of the peptide from the resin must be done by HF, which is highly toxic.

The other main method of SPPS, Fmoc, uses basic conditions for the repetitive removal of the N-alpha-protecting group (Fmoc). Thioesters are not stable to these conditions, and although peptide thioesters from 10 residues to 25 residues have been synthesized by the Fmoc method (see Clippingdale et al. (2000), Li et al. (1998), modified linkers and non-standard Fmoc-deprotection conditions had to be used. Even so, considerable loss of material by premature cleavage of peptide from the resin during Fmoc deprotection makes these resins impracticable for synthesizing longer peptide thioesters.

More recently a "safety-catch" linker has been reported which facilitates the synthesis of thioesters by Fmoc chemistry (Ingenito et al. (1999), Shin et al. (1999)). During the synthesis the linker is inactive to nucleophiles. Following assembly of the peptide chain the linker is activated (so switching off the "safety-catch") and the peptide is cleaved with thiols to form a fully-protected peptide-alpha-thioester. Treatment with TFA and scavengers then affords the fully deprotected peptide-alpha-thioester, which may be purified by standard methods such as RP-HPLC. To date this safety-catch linker has been reported in the synthesis of peptides up to 24 amino acids long (Ingenito et al. (1999), Shin et al. (1999)). However it has been reported that cleavage of a 21 amino acid peptide synthesized on this linker produced very little material (Huse et al. (2000)), and that the desired 47 residue peptide could not be obtained (Marcaurelle et al. (2001)). Whether this linker is stable for producing polypeptides longer than 24 residues is therefore unknown.

In view of the possibilities of native chemical ligation and the sequential ligation approaches described above it seems surprising that there have been few examples of polypeptides over 110 residues that have been synthesized in this way in high purity. The longest published syntheses are those given above by Hackeng et al. (1997) and Hackeng et al. (1999) of the 124-residue human type II secretory phospholipase A2.

Clearly there remain problems in producing long polypeptides (120+ amino acids) of high purity, even by native chemical ligation or sequential native chemical ligation. One difficulty is in the synthesis of long peptide thioesters, particularly by Fmoc chemistry. Boc chemistry has the additional disadvantage that highly toxic HF is used to cleave the peptides from the resin. Another reason is that as peptides become longer they are relatively less soluble, so the kinetics of the ligation step are slow, and thus yields of ligation tend to be lower.

Furthermore, with regard to the interferon proteins the synthesis of the peptide thioesters necessary for native ligation is a challenging task. For example, interferon- $\alpha$ s have four Cys residues, at positions 1, 29, 98 and 137. To synthesize these proteins by one step of native ligation would require the synthesis of a peptide-thioester of 97 amino acids and a peptide of 68 residues. Alternative sites or native ligation would require syntheses of either a peptide thioester or a peptide acid with 137 amino acids, which would be even more difficult to make in practical yield and purity. Even for a synthesis by two steps of native ligation, by ligating IFN(29-97) and IFN(98-165), then IFN(1-28) with the ligated IFN(29-165), it would be necessary to prepare a thioester of 69 amino acids, longer than has ever been reported. The Cys at position 1 in the sequence

introduces another problem, as even for a two step ligation this Cys must be protected during the ligation step and subsequently removed. Following refolding of the polypeptide into the native conformation and oxidation of the Cys to form  
5 the correctly paired disulfide bonds, the active protein must be isolated. As noted above, this is a major problem in the production of the recombinant proteins.

Accordingly, there remains a need in the art for a process to produce interferon proteins that avoids potential  
10 contamination by material present in cell lysates or introduced during protein purification. Furthermore, there is a need for a process which avoids the extensive purification of the native protein that is required in the recombinant process. Further, there is a need in the art for methods of  
15 chemical synthesis which are capable of synthesizing interferons and the long thioesters necessary for their synthesis by native ligation.

It is accordingly an object of the present invention to provide a method for the production of interferon proteins  
20 that avoids the risk of contamination with mouse immunoglobulin, viruses, interferons extended by a Met at the N-terminus and other impurities associated with the production of interferons in cells. It is another object of the invention that following refolding and disulfide bridge  
25 formation interferon with high purity and activity is obtained in one purification step.

#### Summary of the Invention

The present invention provides improved methods for  
30 the production of interferon proteins, comprising one or more cysteine residues and two or more polypeptide subunits, which method comprises the steps of:

(a) synthesizing a first polypeptide subunit of said interferon protein having a Cys, or a ligation auxiliary, as the N-terminal residue by solid-phase peptide synthesis and purifying said subunit;

5 (b) synthesizing at least one additional polypeptide subunit of said interferon protein having a protected Cys or protected auxiliary at the N-terminal and an alpha-carboxylate thioester at the C-terminal by solid-phase peptide synthesis and purifying said additional subunit(s);

10 (c) ligating said first polypeptide subunit and said additional subunit(s) by native chemical ligation to produce a full-length interferon polypeptide;

(d) removing said protective moiety from said interferon polypeptide;

15 (e) oxidizing the cysteine residues in the polypeptide and folding said interferon polypeptide into its native conformation; and

(f) isolating the refolded, biologically active interferon protein.

20 The methods of the invention allow in particular for the solid phase synthesis of two or more fragments of the interferon protein, and takes advantage of the cysteine residues present in interferons to ligate the separate portions to form the full length interferon protein.

25 This invention is based on the unexpected discovery that interferon proteins may be synthesized in high purity and biological activity by chemical synthesis. This method has the advantages of avoiding the problems of N-terminal Met, or contamination with other proteins, murine  
30 immunoglobulins or cellular nucleic acids, all potential risks with interferons produced by recombinant means.

Moreover the inventor has surprisingly found that interferon proteins of high purity and with full biological

activity are obtained in one purification step by reverse-phase HPLC following refolding and disulfide bridge formation.

Furthermore it has surprisingly been found that long  
5 polypeptide fragments of the interferon proteins may be synthesized as C-alpha-thioesters in the high purity necessary for native ligation.

According to preferred aspects of the invention the biologically active interferon protein is selected from the  
10 group consisting of an alpha-interferon protein, a beta-interferon protein, and a gamma-interferon protein. Particularly preferred interferon proteins are interferon alpha2a protein, interferon alpha2b protein, interferon alpha2c protein, synferon, interferon alpha-n1 protein,  
15 interferon alpha-n2 protein, and interferon alpha-n3 protein. The polypeptide subunits are preferably purified using a column chromatography technique with reverse phase column chromatography being preferred.

The polypeptide subunits may be synthesized by  
20 various means known in the art but are preferably produced according to the solid phase synthetic method wherein the N-alpha protective moiety is 9-fluorenylmethyloxycarbonyl (Fmoc).

According to one aspect of the present invention, the  
25 interferon sequence is broken down according to potential sites of ligation, with ligation capable at any cysteine residue. For example, interferon-alpha has four cysteine residues at position 1, 29, 98 and 138. Thus, these four cysteine residues leave open the possibility of linking as  
30 many as four separate portions of full length protein 1-28, 29-97, 98-137, and 138-165 to form the full length interferon-alpha.

According to one embodiment of the present invention Interferon-alpha subunits 1-97 and 98-165 were prepared using conventional solid phase chemistry, cleaved from the resin, ligated together, deprotected and then oxidized to form pure,  
5 full length Interferon-alpha protein.

According to a second embodiment of the present invention interferon-alpha subunits 1-28, 29-97 and 98-165 were prepared using conventional solid phase chemistry, cleaved from the resin and purified. Fragments 29-97 and 98-  
10 165 were ligated together, the N-terminal was deprotected and the resulting fragment 29-165 was ligated with fragment 1-28, Cys1 was deprotected and the full-length interferon-alpha was purified. The interferon-alpha was refolded and the Cys residues were oxidized to form the interferon-alpha native  
15 which was isolated from the refolding mixture to afford the biologically active protein.

According to a third embodiment of the present invention interferon-alpha subunits 1-28, 29-97, 98-137 and 138-165 were prepared using conventional solid phase  
20 chemistry, cleaved from the resin and purified after which the fragments are ligated together, the N-terminal deprotected and the full-length interferon-alpha purified. The interferon-alpha was refolded and the Cys residues were oxidized to form the interferon-alpha native which was  
25 isolated from the refolding mixture to afford the biologically active protein.

According to a fourth embodiment of the present invention interferon-alpha subunits 1-97, 98-137 and 138-165 are prepared using conventional solid phase chemistry,  
30 cleaved from the resin and purified after which the fragments are ligated together, the N-terminal deprotected and the full-length interferon-alpha purified. The interferon-alpha is refolded and the Cys residues are oxidized to form the

interferon-alpha native which is isolated from the refolding mixture to afford the biologically active protein.

Brief description of the figures

5           **Figure 1.** MALDI mass spectrum of interferon-alpha2b synthesized in Example 1.

**Figure 2.** Analytical HPLC of interferon-alpha2b synthesized in Example 1.

10           **Figure 3.** MALDI mass spectrum of interferon-alpha2b synthesized in Example 2.

**Figure 4.** Analytical HPLC of interferon-alpha2b synthesized in Example 2.

**Figure 5.** Amino acid sequence of human interferon-alpha2b. Disulfide bonds are formed between Cys1 and Cys 98,  
15 and between Cys29 and Cys138.

Detailed Description of the Invention

          The present invention provides a solution to the problems of chemical synthesis of alpha-interferons which  
20 present a challenge to the art by virtue of their complexity and size.

          The present invention relates to the production of interferon proteins by use of solid phase peptide synthesis coupled with the native chemical ligation of sections of an  
25 interferon protein to form the full length protein. The target interferon protein is synthesized by utilizing the cysteine residues that are present in the interferon protein. Each cysteine residue present in the target interferon protein is a potential site of ligation, where two sections  
30 of the full length protein can be brought together to make a larger section of the full length interferon protein. This stepwise ligation of sections of the interferon protein will

eventually lead to the formation of the full length interferon protein.

The first step in the method comprises the synthesis of a first section of a full length interferon protein by  
5 standard solid phase peptide synthesis (SPPS). The chemical synthesis of peptides on a solid phase support is for example described by Merrifield (1963), Fields and Noble (1990).

According to the practice of the present invention the synthesis of the first section of the full length protein  
10 is complete when the peptide is elongated to a Cys residue, the chain elongation being made from the C-terminus to the N-terminus. Upon completion of the first section of the interferon protein, the section is cleaved from the resin and purified using standard column chromatography techniques,  
15 including reverse phase column chromatography.

The second polypeptide is then synthesized on a resin from which it may be cleaved to form a peptide thioester, for example according to the procedure of Ingenito et al. (1999). The polypeptide is elongated on this resin by SPPS from the  
20 position preceding the cysteine residue at the N-terminal of the first polypeptide to another Cys. This second Cys bears a protecting group that is stable to the conditions used to remove the protecting groups from the side chains of the other residues, including other Cys in this peptide. Upon  
25 cleavage from the resin, the second section is purified using standard column chromatography techniques, including reverse phase column chromatography.

Upon completion of the individual segments of the interferon protein, the first and second sections of the  
30 target interferon protein are ligated together. This step is for example accomplished using the ligation technique developed by Dawson et al. (1994). The N-terminal Cys of the product from the ligation reaction is then deprotected and

the fully deprotected product is purified using column chromatography, including reverse phase column chromatography.

This sequence of synthesis on the solid phase,  
5 followed by ligation can form the full length interferon protein in only one step of ligation or it can be performed numerous times until the full length protein is formed.

Once the full length protein is synthetically formed, the tertiary structure of the interferon protein is allowed  
10 to be properly developed, and the cysteines are oxidized to form disulfide bonds. The biologically active IFN is then isolated by column chromatography.

It is a preferred embodiment of the invention that the interferon protein is an interferon-alpha. It is a  
15 particularly preferred embodiment of the invention that the interferon protein is an interferon-alpha selected from the group of IFN-alpha2a, IFN-alpha2b, IFN-alpha2c and Synferon.

In one embodiment of the invention the full-length interferon-alpha is assembled by ligating two peptide  
20 fragments comprising IFN-alpha(98-165), having a reduced Cys as the N-terminal residue, with the alpha carboxylate thioester of IFN-alpha(1-97), bearing a protected Cys at the N-terminal.

In another embodiment of the invention the full-  
25 length interferon is assembled by two ligations of three peptide fragments comprising IFN-alpha(1-28), IFN-alpha(29-97) and IFN-alpha(98-165). In this embodiment the fragments comprising IFN-alpha(98-165) and IFN-alpha(29-97) are first ligated and purified to form IFN-alpha(29-165), then a second  
30 ligation is made between IFN-alpha(1-28) and IFN-alpha(29-165) to form IFN-alpha(1-165). Alternatively, the three fragments are IFN-alpha(1-97), IFN-alpha(98-137) and IFN-alpha(138-165).

In a further embodiment of the invention the full-length interferon is assembled by three ligations of four peptide fragments comprising IFN-alpha(1-28), IFN-alpha(29-97), IFN-alpha(98-137) and IFN-alpha(138-165).

5 It is a preferred embodiment of the invention that the polypeptide fragments are synthesized by the method of solid phase peptide synthesis (SPPS). It is particularly preferred that the polypeptide fragments are synthesized by Fmoc SPPS.

10 In preferred embodiments of the invention the group used to protect the N-terminal Cys of the additional polypeptide fragments is selected from the acetamidomethyl (Acm) group, the methylsulfonyl ethoxycarbonyl group (Msc) or the thiazolidine-4-carboxylate residue (Thz).

15 It is a preferred embodiment of the invention that the polypeptides are isolated by reverse phase HPLC.

In another preferred embodiment of the invention the full length, fully deprotected interferon protein is refolded and oxidized by (a) dissolving the fully deprotected  
20 interferon in an aqueous buffer solution with a concentration of a guanidinium salt sufficient to dissolve the interferon polypeptide; (b) diluting the said aqueous buffer solution with another solution containing a redox couple so that the final concentration of the guanidinium salt is 1.5M or less  
25 and the final pH is in the range 5 to 8; and (c) incubating said aqueous buffer solution at a temperature of 20-25°C.

It is a preferred embodiment of the invention that the redox couple is selected from the group of oxidized and reduced glutathione, cysteine and cystine, and oxidized and  
30 reduced cysteamine.

It is a further preferred embodiment of the invention that the refolded and oxidized interferon protein is isolated

as biologically active protein by reverse phase HPLC and lyophilization.

The invention furthermore relates to interferon polypeptide subunit selected from the group consisting of polypeptides comprising the residues 1-28, 29-97, 98-137, 138-165, 1-97, 98-165 of interferon-alpha and to the use of consecutive polypeptide subunits selected from the above group for the production of interferon-alpha.

The present invention will be further illustrated in the examples that follow and that are in no way intended to limit the invention in any way.

## **EXAMPLES**

### **MATERIALS AND METHODS**

#### 15 1. Peptide Synthesis

All peptides were assembled by Solid Phase Peptide Synthesis by the Fmoc method (Fmoc-SPPS). Syntheses were controlled by an Applied Biosystems 433A synthesizer. Boc-Thz-OH was purchased from Bachem (Switzerland). All other amino acids were purchased from Novabiochem (Switzerland).

The following side chain protecting groups were used: Cys with StBu, AcM or Trt; His, Gln and Asn with Trt; Arg with Pmc; Ser, Thr and Tyr with tBu; Glu and Asp with OtBu; and Lys and Trp with Boc.

25 Coupling was carried out with 1 mmol DIC-HOBt and 1 mmol Fmoc amino acid per coupling in dimethylformamide (DMF) - N-Methylpyrrolidine (NMP) and Fmoc deprotection was by 20% piperidine in DMF.

Capping of unreacted amine groups after each coupling step was carried out by acetic anhydride-ethyldiisopropylamine in N,N-dimethylformamide (DMF).

Following completion of the automated synthesis the resin was washed with DMF and isopropanol and dried under vacuum.

5    2.    Peptide purification

Unless stated otherwise, purification of peptides was carried out by reverse-phase HPLC. Preparative HPLC was done using a C8 Vydac column (250 x 22 mm, 10 mm particle size, 208TP1022) at 10 ml/min eluting with a linear gradient of  
10    acetonitrile-0.1% trifluoroacetic acid in water-0.1% trifluoroacetic acid, controlled by a Waters semi-prep HPLC system. The purification was monitored by UV at 214nm.

Fractions were analyzed by MALDI-MS and those containing the target polypeptide were combined and  
15    lyophilized to afford the desired peptide. If necessary, fractions were repurified by the same method.

Semi-preparative RP-HPLC was carried out on the same system and with the same solvents, eluting through a Vydac C8 column (250 x 10 mm, 5 mm particle size, 208TP510) at 3  
20    ml/min:

3.    Analysis of peptides

The purity of the peptides was analysed on a Vydac C18 column (238TP54) eluting with a linear gradient of  
25    acetonitrile-0.09% trifluoroacetic acid in water-0.1% trifluoroacetic acid, controlled by a Waters Alliance HPLC system. The analysis was monitored at 214nm. MALDI-MS was performed on a Voyager™ Elite Biospectrometry™ Research Station (PerSeptive Biosystems).

30

**EXAMPLE 1**

Production of human interferon-alpha2b on the basis of two interferon-alpha subunit polypeptides

## 1. Introduction

According to this Example, human interferon-alpha2b was produced according to the method wherein two interferon-alpha subunit polypeptides were prepared by solid-phase peptide synthesis. The approach taken in this Example was the synthesis of two large sections of the full length interferon-alpha protein, wherein after one ligation reaction the full length human interferon-alpha protein was formed.

Thus, a polypeptide comprising amino acids 1-97 of human interferon-alpha was synthesized using Fmoc-SPPS. The other segment of interferon-alpha, from position 98-165, was also prepared using Fmoc-SPPS. According to the present Example, the cysteine residue at the 98 position of interferon-alpha was utilized to ligate the two sections together to form the full length human interferon-alpha, which was then allowed to reform the native, biologically active structure and the native disulfide bonds and isolated.

## 2. Preparation of interferon-alpha2b(98-165)

Interferon-alpha (98-165) comprises a 68 amino acid section of human interferon-alpha from position 98 to position 165 in the amino acid sequence of interferon-alpha holoprotein. Interferon-alpha (98-165) was prepared by solid-phase peptide synthesis (SPPS) on Fmoc-Glu(OtBu)-Novasyn® TGA resin, initial loading 0.13mmol/g, on a scale of 0.08 mmol.

Peptide was cleaved from the resin (0.67g of peptide-resin, one half of the synthesis) with a mixture of TFA-triisopropylsilane-water-phenol-ammonium iodide (21 ml, 3 ml, 1.25 ml, 1.25 g, 0.40 g) for 3 hours. The resin was removed by filtration and the peptide was precipitated by addition of the cleavage solution to cold methyl t-butyl ether (3 tubes of 35 ml).

The precipitate was pelleted by centrifugation and washed three times with cold methyl t-butyl ether (2 x 30 ml and 1 x 20 ml per tube). This afforded a white powder which was lyophilized from acetic acid-water. The crude material  
5 was purified by semi-preparative RP-HPLC. After lyophilization interferon-alpha2b(98-165) was obtained, 1.9 mg, 77% purity by analytical HPLC.

3. Preparation of interferon-alpha2b(1-97)Cys1(Acm) ethyl-3-mercaptopropionate thioester  
10

Interferon-alpha(1-97)Cys1(Acm) ethyl-3-mercaptopropionate thioester comprises the first 97 amino acids of human interferon-alpha2b (Fig. 5; SEQ ID NO: 1) wherein the terminal carboxyl group at position 97 has been converted to  
15 an ethyl-3-mercaptopropionate thioester. The C-terminal amino acid, Ala, was coupled to 4-sulfamylbutyryl AM resin (Novabiochem) by the method of Backes and Ellman (1999). Thus, Fmoc-Ala-OH (6.0 g) and ethyldiisopropylethylamine (6.2 ml) were added to a suspension of 4-sulfamylbutyryl AM resin  
20 (3.28 g, initial loading 1.12 mmol/g) in chloroform (35 ml). PyBOP® (1.56 g) and chloroform (5 ml) were added and stirring was continued at -18°C for 1.5 hours.

The resin was filtered and washed with chloroform, isopropanol and dried under high vacuum. Unreacted  
25 sulfonamide groups were capped with a solution of di-t-butyl dicarbonate (1.5 g) and ethyldiisopropylamine (2.4 ml) in dichloromethane (DCM, 2 x 15 min). After each capping the resin was washed with dichloromethane and after the second it was dried under high vacuum.

30 The loading of Fmoc-Ala was measured as 0.38mmol/g using the method described in the Novabiochem 2000 Catalogue for Solid Phase Peptide Synthesis, page P4. This resin was

used as the starting resin for the solid-phase synthesis on a scale of 0.1 mmol.

Protocols and reagents were the same as for the synthesis of interferon-alpha2b(98-165), with the following exceptions. Ser68-Thr69 and Ile24-Ser25 were coupled as the pseudoproline dipeptides. Half of the resin was removed from the reaction vessel after the coupling of Lys31. The synthesis was continued and the final residue was coupled as Fmoc-Cys(Acm), Fmoc-deprotected and reprotected with the Boc group as described above. The resin was washed and dried under high vacuum, weight 0.37 g.

4. Cleavage of interferon-alpha2b(1-97)-cys1(Acm) ethyl-3-mercaptopropionate thioester

The thioester of interferon-alpha2b(1-97), prepared above, was then cleaved from the resin using the method of Ingenito et al. (1999). Thus a flame-dried, 100 ml round bottom flask was charged with argon and Boc-protected peptide resin (0.37 g) and anhydrous tetrahydrofuran (THF, 10 ml) were added. After 10 min. trimethylsilyldiazomethane (10 ml, 2M solution in hexanes) was added and the mixture was stirred at ambient temperature for 2 hours. The resin was separated by filtration and washed with THF and DCM. Subsequently the resin was placed in a 50 ml round bottom flask. Subsequently the resin was placed in a 50 ml round bottom flask and the flask was charged with argon. DMF (anhydrous, 7 ml), ethyl-3-mercaptopropionate (2 ml) and a solution of thiophenol sodium salt (66 mg in 1 ml anhydrous DMF) were added by syringe and the mixture was gently stirred under argon for 20 hours.

The volatiles were removed under high vacuum and the residue was stirred with TFA-triisopropylsilane-water-phenol-ammonium iodide (10.5 ml, 1.5 ml, 0.6 ml, 0.6 g, 0.25 g) at room temperature for 3 hours. The resin was removed by

filtration and the solution was added to cold methyl t-butyl ether (MTBE). The white precipitate was pelleted by centrifugation, washed three times with MTBE and the precipitate obtained was dissolved in 50-50 acetonitrile-  
5 water and lyophilized. This crude material was purified by preparative RP-HPLC. Fractions were analyzed by MALDI-MS and those containing the target polypeptide were combined and lyophilized.

If necessary, fractions were repurified by the same  
10 method. Interferon-alpha2b(1-97)-cys1(Acm) ethyl-3-mercaptopropionate thioester was obtained in 96% purity by analytical HPLC, 2.8 mg.

#### 5. Preparation of interferon-alpha2b-Cys1(Acm)

15 The ligation reaction proceeded with the reaction of near equi-molar amounts of the interferon-alpha(1-97)Cys1(Acm) ethyl-3mercaptopropionate thioester and interferon-alpha(98-165). Interferon-alpha(1-97)Cys1(Acm) ethyl-3mercaptopropionate thioester, 2.84 mg, and interferon-  
20 alpha(98-165), 1.9 mg, were dissolved in separate solutions of 6M guanidinium chloride, 0.1M sodium phosphate pH 7.5 (0.12 ml per peptide). The two solutions were combined and thiophenol, 2.4 µl, was added and the solution was agitated gently. Further thiophenol was added after 16 hours (2.4 µl)  
25 and 24 hours (4.8 µl). After 42 hours dithiothreitol (DTT, 120mg) was added. After 44 hours the solution was diluted with 3 ml of 1:2 acetonitrile:6M guanidinium chloride and purified by semi-preparative RP-HPLC.

Fractions were analyzed by MALDI-MS and those  
30 containing the target polypeptide were combined and lyophilized to afford interferon-alpha2b-Cys1(Acm), 1.07 mg.

6. Preparation of interferon-alpha2b by deprotection of Cys1(Acm)

Interferon-alpha2bCys1(Acm), 1.07 mg, was dissolved in 0.2 ml of 50% acetic acid-water. A solution of 0.8 mg of  
5 mercuric acetate in 15  $\mu$ l of 50% acetic acid-water was added and the solution was sparged with argon. The solution was agitated gently for 6 hours.  $\beta$ -Mercaptoethanol (18  $\mu$ l) was added and the solution was agitated for a further 23 hours. The mixture was centrifuged and the supernatant was purified  
10 by RP-HPLC on a Vydac C18 column (238TP54), eluting with a linear gradient of acetonitrile-0.1% TFA in water-0.1% TFA, controlled by a Waters Alliance system. The purification was monitored by UV at 214nm and fractions of the major peak were analyzed by MALDI-MS. Those fractions containing the desired  
15 peptide were combined and lyophilized to afford 0.81 mg.

7. Folding of interferon-alpha2b

The synthetic interferon-alpha was allowed to fold into the biologically active interferon-alpha2b in an  
20 oxidative buffer comprising glutathione oxidized:reduced in a ratio of 1:2. Thus, 0.34 mg of deprotected interferon-alpha2b from paragraph 6 was dissolved in 0.22 ml of buffer A (0.1M Tris, 6M GdmCl, 1mM EDTA sodium salt, pH 7.5) that had been sparged with argon. This solution was diluted with 30 ml each  
25 of solutions of oxidized glutathione and of reduced glutathione, each solution containing 0.3 mg of glutathione in 30  $\mu$ l of buffer B (0.1M Tris, 1 mM EDTA sodium salt, pH 7.5), and with 0.66 ml of buffer B. Samples were removed for analysis by RP-HPLC at the start of the folding and after 22  
30 hours. The elution time of the main peak changed from 40.2 minutes at the start to 36.3 minutes after 22 hours, consistent with folding of the protein.

After 22 hours the folding mixture was centrifuged at 13000 rpm for 1 min. No pellet was observed and the solution was desalted on a Hi-Trap™ column (Pharmacia), controlled by a KTa FPLC, eluting with PBS pH 7.

5       The purification was monitored by UV at 280 nm and the most concentrated fraction was used for analysis of biological activity. The concentration was measured at  $0.09 \pm 0.03$  mg/ml by the Advanced Protein Assay (Cytoskeleton Inc.). A preparation that was folded in a similar way was purified  
10 by RP-HPLC on a C18 column and the main peak was isolated and lyophilized.

MALDI-MS of this sample showed that a peptide with the expected mass was present and an NEM test for free Cys residues (Mant et al. (1997)) was negative, confirming that  
15 both disulfide bonds had formed. The purity was 95% by analytical HPLC.

#### 8. Test of inhibition of cytopathic affect by interferon-alpha2b

20       Interferon-alpha2b prepared in paragraph 7 was titrated using the cytopathic effect inhibition assay of Rubinstein et al. (1981). In this antiviral assay one unit/ml of interferon is the quantity necessary to produce a cytopathic effect of 50% on vesicular stomatitis virus (VSV)  
25 with bovine MDBK cells. The most concentrated fraction of interferon-alpha2b prepared in paragraph 7 was diluted by  $1 \times 10^4$  with DMEM and added to bovine MDBK cells (0.18 ml interferon-alpha was added to 0.15 ml of cells at  $5 \times 10^4$  cells /ml) in a 96-well plate. Aliquots of interferon added  
30 to each well were a third of the concentration of the interferon in the previous well. After 24 hours the cells were challenged with VSV. 24 hours after challenge the cells were examined by microscope and the well in which 50% of

cells survived was taken as having a concentration of one unit of interferon/ml. The activity of interferon-alpha2b measured by this test was calculated to be  $2.5 \pm 0.8 \times 10^7$  U/mg.

5

**EXAMPLE 2**1. Preparation of interferon-alpha2b(98-165)

This synthesis was performed as described in Example 1.

10

2. Preparation of interferon-alpha2b(29-97)Cys29Thz ethyl-3-mercaptopropionate thioester

Interferon-alpha2b(29-97)Cys29Thz ethyl-3-mercaptopropionate thioester comprises the amino acids 29-97 of human interferon-alpha2b wherein the terminal carboxyl group at position 97 has been converted to an ethyl-3-mercaptopropionate thioester and the cysteine residue at position 29 has been incorporated as the (L)-thiazolidine-4-carboxylic acid (Thz) residue. The C-terminal amino acid, Ala, was coupled to 4-sulfamylbutyryl AM resin (Novabiochem) by the method of Backes and Ellman (1999) as described for the synthesis of interferon-alpha2b(1-97)Cys1(Acm) ethyl-3-mercaptopropionate thioester in Example 1. This resin was used as the starting resin for the solid-phase synthesis on a scale of 0.1 mmol. Protocols and reagents were the same as for the synthesis of interferon-alpha2b(1-97)Cys1(Acm) ethyl-3-mercaptopropionate thioester except that the synthesis was finished at Cys29Thz by coupling the final residue as Boc-Thz-OH (Boc-L-thiazolidine-4-carboxylic acid). The resin was washed and dried under high vacuum, 1.07 g.

3. Cleavage of interferon-alpha2b(29-97)Cys29Thz ethyl-3-mercaptopropionate thioester

The peptide interferon-alpha2b(29-97)Cys29Thz ethyl-3-mercaptopropionate thioester was cleaved from the resin using the method of Ingenito et al. (1999). Thus, a flame-dried, 100ml round bottom flask was charged with argon and Boc-protected peptide resin (0.50 g) and anhydrous tetrahydrofuran (THF, 7 ml) were added. After 10 min trimethylsilyldiazomethane (7 ml, 2M solution in hexanes) was added and the mixture was stirred at ambient temperature for 2 hours. The resin was separated by filtration and washed with THF and DCM. Subsequently the resin was placed in a 50 ml round bottom flask and the flask was charged with argon. DMF (anhydrous, 7 ml), ethyl-3-mercaptopropionate (2 ml) and a solution of thiophenol sodium salt (65 mg in 1 ml anhydrous DMF) were added by syringe and the mixture was gently stirred under argon for 20 hours.

The volatiles were removed under high vacuum and the residue was treated with TFA-triisopropylsilane-water-phenol-ammonium iodide at room temperature for 3 hours. The resin was removed by filtration and the solution was added to cold methyl t-butyl ether to precipitate the peptide. The white precipitate was pelleted by centrifugation, washed three times with cold MTBE and the resulting white precipitate was dissolved in 50-50 acetonitrile-water and lyophilized. This crude material was purified by preparative RP-HPLC.

Fractions were analyzed by MALDI-MS and those containing the target polypeptide were combined and lyophilized to afford 13.9 mg. The purity measured by analytical RP-HPLC was 82%.

4. Preparation of interferon-alpha2b(1-28)Cys1Thz ethyl-3-mercaptopropionate thioester

Interferon-alpha(1-28)Cys1Thz ethyl-3-mercapto-propionate thioester comprises the first 28 amino acids of human interferon-alpha2b wherein the terminal carboxyl group at position 28 has been converted to an ethyl-3-mercaptopropionate thioester. The C-terminal amino acid, Ser, was coupled to 4-sulfamylbutyryl AM resin (Novabiochem) by the method of Backes and Ellman (1999). Thus, Fmoc-Ser(tBu)-OH (4.17 g) and ethyldiisopropylethylamine (3.7 ml) were added to a suspension of 4-sulfamylbutyryl AM resin (1.95 g, initial loading 1.12 mmol/g) in chloroform (20 ml). The mixture was cooled to -20°C, PyBOP® (5.67g) was added and stirring was continued at -20°C for 3.5 hours. The resin was filtered and washed with chloroform. Unreacted sulfonamide groups were capped with a solution of di-t-butyl dicarbonate (1.5 g) and ethyldiisopropylamine (2.4 ml) in dichloromethane (DCM, 2 x 15 min).

After each capping the resin was washed with dichloromethane and after the second it was dried under high vacuum. The loading of Fmoc-Ser(tBu) was measured as 0.46 mmol/g using the method described in the Novabiochem 2000 Catalogue for Solid Phase Peptide Synthesis, page P4. This resin was used as the starting resin for the solid-phase synthesis on a scale of 0.1 mmol.

Protocols and reagents were the same as for the synthesis of interferon-alpha(98-165) with the following exceptions. Ile24-Ser25 were coupled as the c-pseudoproline dipeptides. The residues Arg12, Arg13, Thr14, Leu15, Met16, Leu17, Leu18, Ala19, Arg22 and Arg23 were all double-coupled. Cys29 was incorporated as B(L)-thiazolidine-4-carboxylic acid (Thz). The synthesis was finished at Cys1Thz by coupling the

final residue as Boc-Thz-OH. The resin was washed and dried under high vacuum, weight 0.37 g.

5. Cleavage of interferon-alpha2b(1-28)Cys1Thz ethyl-3-mercaptopropionate thioester

The thioester of interferon-alpha2b(1-28), prepared above, was then cleaved from the resin using the method of Ingenito et al. (1999). Thus a flame-dried, 50 ml round bottom flask was charged with argon and Boc-protected peptide resin (0.18 g) and anhydrous tetrahydrofuran (THF, 7.5 ml) were added. After 10 min trimethylsilyldiazomethane (7.5 ml, 2M solution in hexanes) was added and the mixture was stirred at ambient temperature for 2 hours. The resin was separated by filtration and washed with THF and DCM. Subsequently the resin was placed in a 50 ml round bottom flask and the flask was charged with argon. DMF (anhydrous, 7 ml), ethyl-3-mercaptopropionate (2 ml) and a solution of thiophenol sodium salt (65 mg in 1 ml anhydrous DMF) were added by syringe and the mixture was gently stirred under argon for 20 hours. The volatiles were removed under high vacuum and the residue was stirred with TFA-phenol-triisopropylsilane-water-ammonium iodide at room temperature for 3 hours.

The resin was removed by filtration and the solution was added to cold methyl t-butyl ether (MTBE) to precipitate the peptide. The white precipitate was pelleted by centrifugation, washed three times with cold MTBE and the resulting white precipitate was dissolved in 50-50 acetonitrile-water and lyophilized. This crude material was purified by RP-HPLC on a Vydac C8 column eluting with a linear gradient of acetonitrile-0.1% TFA in water-0.1% TFA, controlled by a Waters semi-prep HPLC system. The purification was monitored by UV at 214 nm.

Fractions were analyzed by MALDI-MS and those containing the target polypeptide were combined and lyophilized to afford 17.0 mg of the desired product of purity 91% by analytical HPLC.

5

6. Preparation of interferon-alpha2b(29-165)Cys29Thz

The ligation reaction proceeded with the reaction of a near equi-molar amounts of the interferon-alpha2b(29-97)Cys29Thz ethyl-3-mercaptopropionate thioester and  
10 interferon-alpha2b(98-165). Interferon-alpha2b(29-97)Cys29Thz ethyl-3mercaptopropionate thioester, 10.6 mg, and interferon-alpha2b(98-165), 13.7 mg, were dissolved in separate solutions of 6M guanidinium chloride, 0.1M sodium phosphate pH 7.5 (0.60 ml per peptide). The two solutions were mixed,  
15 thiophenol, 24 µl, and benzyl mercaptan, 24 µl, were added and the solution was incubated at 37°C.

After 66 hours dithiothreitol (DTT, 0.37 g) was added as a solution in 3 ml of 6M guanidinium chloride, 0.1M sodium phosphate pH 7.5 and the ligation mixture was incubated for a  
20 further 2 hours at 37°C. The solution was diluted with 3 ml acetonitrile and 2 ml 6M guanidinium chloride, 0.1M sodium phosphate pH 7.5 and purified by RP-HPLC on a Vydac C8 column (208TP510) with a linear gradient of acetonitrile-0.1% TFA in water-0.1% TFA, controlled by a Waters semi-prep HPLC system.  
25 The purification was monitored by UV at 214 nm. Fractions were analysed by MALDI-MS and those containing the target polypeptide were combined and lyophilized to afford 4.7 mg, purity 97% by analytical HPLC.

30 7. Preparation of interferon-alpha2b(29-165) by conversion of Thz29 to Cys

The N-terminal Cys of interferon-alpha2b(29-165)Cys29Thz was regenerated by treatment with O-methyl-

hydroxylamine hydrochloride at pH 4.0. Interferon-alpha2b(29-165)Cys29Thz (4.6mg) was dissolved in 6M guanidinium hydrochloride, 0.3M O-methylhydroxylamine hydrochloride, 0.1M acetic acid adjusted to pH 4.0 with sodium hydroxide. After  
5 0.5 hours at 22-24°C the solution was incubated at 37°C. The solution was desalted on a Hi-Trap™ column (Pharmacia), controlled by a KTa FPLC, eluting with 6M guanidinium chloride 0.1M sodium phosphate pH 7.0.

The purification was monitored by UV at 280nm and the  
10 first peak (1.8 ml) was concentrated to 0.4 ml in a Centricon cartridge (Millipore, molecular weight cut-off 10 kDa). This solution was used immediately in the ligation with interferon-alpha2b(1-28)Cys1Thz (example 2, paragraph 8).

15 8. Preparation of interferon-alpha2b(1-165)

The ligation reaction proceeded with the reaction of near equimolar amounts of the interferon-alpha2b(29-97)Cys29Thz ethyl-3mercaptopropionate thioester and interferon-alpha2b(98-165). Interferon-alpha2b(1-28)Cys1Thz,  
20 0.8mg, was dissolved in 6M guanidinium chloride, 0.1M sodium phosphate pH 7.0 (0.1 ml) and added to the concentrated solution of interferon- alpha2b(29-165) obtained from the HiTrap™ (estimated to be 4.0 mg). Thiophenol, 10 µl, and benzyl mercaptan, 10 µl, were added, the solution was mixed  
25 and incubated at 37°C. After 36 hours dithiothreitol (DTT, 15.7 g) was added as a solution in 6M guanidinium chloride, 0.1M sodium phosphate pH 7.0 (0.1 ml) and the ligation mixture was incubated at 37°C. After 1.5 hour O-methylhydroxylamine hydrochloride(8.7mg) was added as a  
30 solid, the ligation mixture was diluted with 4 ml a solution of 6M guanidinium hydrochloride, 0.3M O-methylhydroxylamine hydrochloride, 0.1M acetic acid adjusted to pH 4.0 with sodium hydroxide, acetonitrile (0.2 ml) and trifluoroacetic

acid (5  $\mu$ l) and incubated at 37°C. After a further 3 hours the solution was purified by RP-HPLC and fractions containing the desired peptide were pooled and lyophilized to afford 0.89 mg of reduced interferon-alpha2b(1-165), 92% purity by analytical HPLC.

#### 9. Folding of interferon-alpha2b

The synthetic interferon-alpha2b was allowed to fold into the biologically active interferon-alpha. Thus, interferon-alpha2b (0.88 mg) was dissolved in 0.5 ml of buffer A (0.1M Tris, 6M GdmCl, 1mM EDTA sodium salt, pH 8.0) that had been sparged with argon. This solution was diluted with 61  $\mu$ l each of solutions of oxidized glutathione and of reduced glutathione, each solution containing 0.61 mg of glutathione in 61  $\mu$ l of buffer B (0.1M Tris, 1 mM EDTA sodium salt, pH 8.0), and with 1.5 ml of buffer B. The solution was mixed and allowed to stand at room temperature (22°C). After 16 hours the folded protein was purified by RP-HPLC on a Vydac C18 column (238TP54), samples were pooled and lyophilized to afford native IFN-alpha2b, 0.15mg, 100% purity by analytical HPLC. An NEM test for free Cys confirmed that none was present.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

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**CLAIMS**

1. A method of producing a biologically active interferon protein, comprising one or more cysteine residues and two or more polypeptide subunits, which method comprises the steps of:

5 (a) synthesizing a first polypeptide subunit of said interferon protein having a Cys, or a ligation auxiliary, as the N-terminal residue by solid-phase peptide synthesis and purifying said subunit;

10 (b) synthesizing at least one additional polypeptide subunit of said interferon protein having a protected Cys or protected auxiliary at the N-terminal and an alpha-carboxylate thioester at the C-terminal by solid-phase peptide synthesis and purifying said additional subunit(s);

15 (c) ligating said first polypeptide subunit and said additional subunit(s) by native chemical ligation to produce a full-length interferon polypeptide;

(d) removing said protective moiety from said interferon polypeptide;

20 (e) oxidizing the cysteine residues in the polypeptide and folding said interferon polypeptide into its native conformation; and

(f) isolating the refolded, biologically active interferon protein.

25 2. The method as claimed in claim 1, wherein said biologically active interferon protein is selected from the group consisting of an alpha-interferon protein, a beta-interferon protein, and a gamma-interferon protein.

30 3. The method as claimed in claim 2, wherein said biologically active alpha-interferon protein is selected from the group consisting of interferon alpha-2a protein, interferon alpha-2b protein, interferon alpha-2c protein,

synferon, interferon alpha-n1 protein, interferon alpha-n2 protein, and interferon alpha-n3 protein.

4. The method as claimed in any one of the claims 1-3, wherein said first polypeptide subunit comprises residues 98-165 of interferon-alpha.

5. The method as claimed in any one of the claims 1-4, wherein said additional polypeptide subunit comprises residues 1-97 of interferon-alpha.

6. The method as claimed in any one of the claims 1-4, wherein said additional polypeptide subunits comprise residues 29-97 and residues 1-28 of interferon-alpha.

7. The method as claimed in any one of the claims 1-3, wherein said first polypeptide subunit comprises residues 138-165 of interferon-alpha and said additional polypeptide subunits comprise residues 1-97 and residues 98-137 of interferon-alpha.

8. The method as claimed in any one of the claims 1-3, wherein said first polypeptide subunit comprises residues 138-165 of interferon-alpha and said additional polypeptide subunits comprise residues 1-28, residues 29-97 and residues 98-137 of interferon-alpha.

9. The method as claimed in any one of the claims 1-8, wherein said first polypeptide subunit is purified using a column chromatography technique.

10. The method as claimed in claim 9, wherein said column chromatography technique is reverse phase column chromatography.

11. The method as claimed in any one of the claims 1-10, wherein said additional polypeptide subunit(s) are purified using a column chromatography technique.

12. The method as claimed in claim 11, wherein said column chromatography technique is reverse phase column chromatography.

13. The method as claimed in any one of the claims 1-12, wherein the peptides are synthesized by solid-phase peptide synthesis.

14. The method as claimed in claim 13, wherein the  
5 peptides are synthesized by Fmoc solid-phase peptide synthesis.

15. The method as claimed in any one of claims 1-14, wherein the group protecting the Cys or auxiliary at the N-terminal of the additional subunit(s) is selected from the  
10 group consisting of acetamidomethyl (Acm), methylsulfonylethyloxycarbonyl (Msc), thiazolidine (Thz), methylbenzyl, methoxybenzyl, picolyl (Pic), benzyl (Bzl), tert-butylthio (StBu) and t-butyl (tBu).

16. The method as claimed in any one of the claims 1-  
15 15, wherein the folding of said interferon polypeptide into its native conformation to form a biologically active interferon protein is done by

(a) dissolving the said polypeptide in an aqueous buffer solution comprising 6M guanidinium salt, 0.1M  
20 phosphate buffer, 1mM EDTA sodium salt; and

(b) diluting the said aqueous buffer solution with another solution containing oxidized and reduced glutathione such that the final concentration of the guanidinium salt is 1.5M, the final concentration of phosphate is 0.1M, the final  
25 concentration of EDTA is 1 mM, the final concentration of oxidized glutathione is 0.5mM and the final concentration of reduced glutathione is 1mM.

17. The method as claimed in any one of the claims 1-16, wherein the interferon protein is isolated by reverse  
30 phase column chromatography and lyophilization.

18. The method as claimed in any one of the claims 1-17, wherein the peptide thioesters are synthesized by Fmoc chemistry on a resin with a 4-sulfamylbutyryl linker.

19. The method as claimed in claim 18, wherein the peptide thioesters are cleaved from the resin in a two-step procedure using

(a) diazomethane or (trimethylsilyl)diazomethane to  
5 activate the linker; and

(b) the sodium salt of thiophenol to displace the polypeptide from the linker.

20. Interferon polypeptide subunit selected from the group consisting of polypeptides comprising the residues 1-  
10 28, 29-97, 98-137, 138-165, 1-97, 98-165 of interferon-alpha.

21. Use of consecutive polypeptide subunits selected from the group consisting of polypeptides comprising the residues 1-28, 29-97, 98-137, 138-165, 1-97, 98-165 for the production of interferon-alpha.