UNITED STATES DISTRICT COURT
DISTRICT OF MASSACHUSETTS

AMGEN INC., Plaintiff, v. F. HOFFMANN-LA ROCHE LTD, ROCHE DIAGNOSTICS GmbH, and HOFFMANN-LA ROCHE INC. Defendants.	CIVIL ACTION No.: 05-CV-12237WGY

SUPPLEMENTAL DECLARATION OF THOMAS R. KADESCH, PH.D. IN FURTHER SUPPORT OF DEFENDANTS' PROPOSED CLAIM CONSTRUCTION

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Counsel for Defendants,
F. HOFFMANN-LA ROCHE LTD,
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HOFFMANN-LA ROCHE INC.

I, Thomas R. Kadesch, Ph.D., hereby declare as follows:

I make this declaration in connection with Defendants' Opposition to Amgen, Inc.'s Claims Construction Brief in the above captioned action.

I. INFORMATION CONSIDERED IN FORMING MY OPINIONS

1. This expert declaration is provided to assist the Court in understanding the science and technology discussed in Defendants' Opposition. In forming the opinions set forth in this expert declaration, I have considered U.S. Patent No. 4,703,008; and the claims of U.S. Patent Nos. 5,547,933; 5,441,868; 5,618,698; 5,756,349; 5,955,422 and 5,621,080; (collectively the Lin Patents; Exhibit 2 to Kadesch Decl.), in the context of my knowledge of relevant literature and my academic and professional experience.

II. ERYTHROPOIETIN

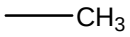
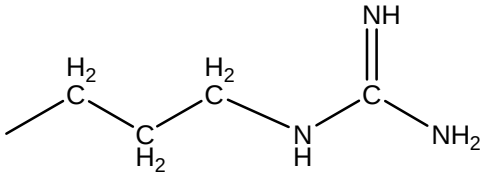
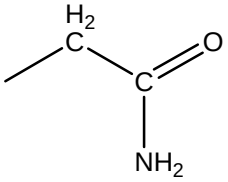
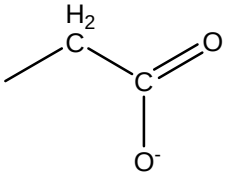
2. Human erythropoietin is a 34,000 dalton glycoprotein hormone. When produced by a mammalian cell from a gene encoding human erythropoietin the secreted polypeptide has 165 specific amino acid residues. Figure 9 of the Lin Patents identifies each of the amino acid residues of the human (and monkey) erythropoietin polypeptide by an art-recognized single letter abbreviation. Figure 6 of the Lin Patents identifies the specific amino acid residues in the human erythropoietin polypeptide by an art-recognized three-letter abbreviation. (See Kadesch Decl. Ex. 3 ; Table 2-1 Abbreviations for amino acids). The first amino acid residue of the mature human erythropoeitin sequence is designated "+1" in these figures.

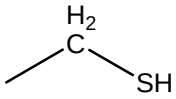
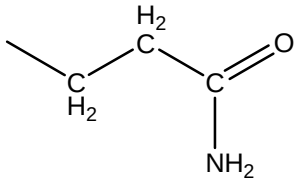
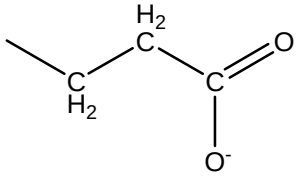
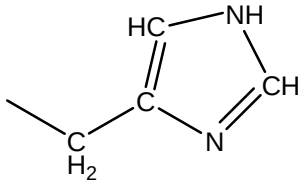
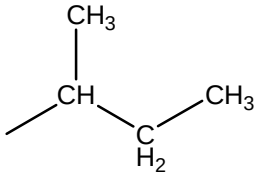
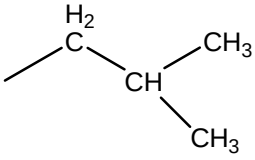
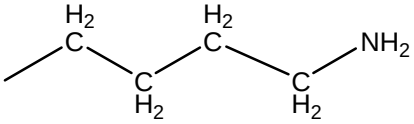
3. Example 5 of the Lin Patents contains a statement that

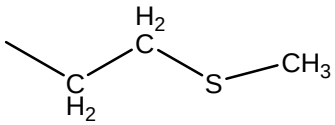
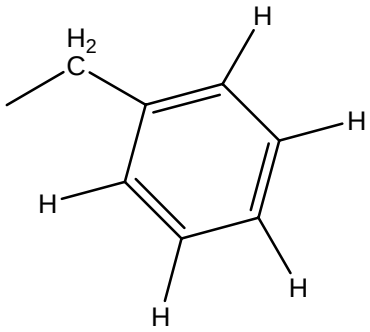
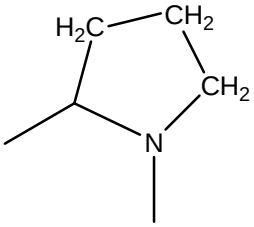
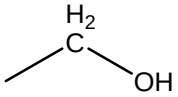
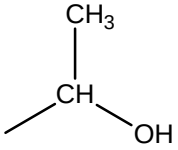
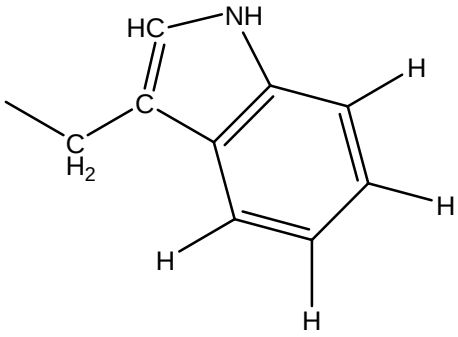
> FIG 6 thus serves to identify the primary structural conformation (amino acid sequence) of mature human EPO as including 166 specified amino acid residues (estimated M.W. = 18,399) . . . Sites for potential glycosylation of the mature human EPO polypeptide are designated in the Figure by asterisks.

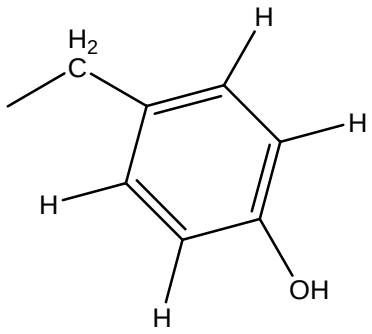
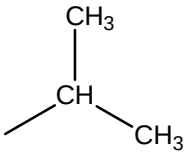
('008 Patent col.21 l.61 - col.22 l.2).

4. By inspection of the sequences in either Figure 6 or Figure 9, starting at residue +1 and proceeding through residue +165, one can count the number of times each type of amino acid residue occurs in the sequence. The table below shows the number of times each of the twenty naturally occurring amino acids appears as a residue in the structure of mature human erythropoietin and the canonical structural formula of the side chain group which defines the residue.

	AA Residue	Abbreviations	Side Chain Structural Formula	Occurrences in Human EPO
1.	Alanine	A, Ala		19
2.	Arginine	R, Arg		12
3.	Asparagine	N, Asn		6
4.	Aspartic acid	D, Asp		6

5.	Cysteine	C, Cys		4
6.	Glutamine	Q, Gln		7
7.	Glutamic acid	E, Glu		12
8.	Glycine	G, Gly		9
9.	Histidine	H, His		2
10.	Isoleucine	I, Ile		5
11.	Leucine	L, Leu		23
12.	Lysine	K, Lys		8

13.	Methionine	M, Met		1
14.	Phenylalanine	F, Phe		4
15.	Proline	P, Pro		8
16.	Serine	S, Ser		10
17.	Threonine	T, Thr		11
18.	Tryptophan	W, Trp		3

19.	Tyrosine	Y, Tyr		4
20.	Valine	V, Val		11

5. According to the teaching of the Lin Patents and what is understood by a person of skill in the art, whenever a mammalian cell expresses a gene encoding human erythropoietin a polypeptide is produced having the side chains specified in the table. Each and every atom shown contributes to the essential structural attributes of erythropoietin expressed by a mammalian host cell.

6. A mammalian host cell that produces erythropoietin from a gene encoding the human sequence will modify the side chains of only three specific asparagine residues and one specific serine residue. By a natural cellular process catalyzed by an enzyme, a hydrogen atom is removed from the side chain amide of asparagine (Asn) at positions Asn24, Asn38, and Asn83 and from the side chain hydroxyl of serine (Ser) at position Ser126. A new bond to a sugar residue is formed in place of the lost hydrogen. This type of post-translational modification is known as glycosylation. (Ex. 4). The Lin Patents expressly describe potential glycosylation of the asparagines, but not the serine.

7. The cysteine (Cys) residues at positions Cys7 and Cys161 can each lose a hydrogen and form an intramolecular disulfide, an S-S bond, as can the cysteine residues at positions Cys29 and Cys33. (Ex. 4). The S-S bond formation process is reversible and dependent on the particular conditions present. Disulfides are not fully described in the Lin Patents.

8. As mentioned above, secreted human erythropoietin produced by mammalian cells is 165 amino acid residues. The sequence is the result of truncation of a longer sequence. The C-terminal arginine, Arg166, is cleaved from the premature polypeptide by proteolytic enzymes making the final residue of the mature sequence aspartic acid (Asp165). (Ex. 5). This cleavage is not reported in the Lin Patents. On the N-terminus, 27 amino acid residues are cleaved by the mammalian host cell from the premature sequence, making the first amino acid residue of the mature sequence alanine (Ala1).

9. Glycosylation, truncation of the pre-mature sequence, and disulfide bond formation are the post-translational events that occur in the production of mature erythropoietin produced in mammalian cells expressing a gene encoding human erythropoietin, either known at the time of the patent filing date or subsequently discovered.

10. All other atoms of the amino acid residues, including all side chain atoms are conserved and thus define the structure of erythropoietin produced from mammalian cells expressing a gene encoding for human erythropoietin. The N-terminal residue of erythropoietin produced by mammalian cells always has a free amino group ($-NH_2$) which is positively charged under physiological conditions ($-NH_3^+$). The side chains of the residues derived from lysine (Lys) at positions Lys20, Lys45, Lys52, Lys97, Lys116, Lys140, Lys152, and Lys154 always

have four methylene groups (-CH_2-) attached to a primary amino group (-NH_2). The primary amino groups are also positively charged (-NH_3^+) under physiological conditions.

III. FURTHER TESTIMONY

11. If requested by the Court I may provide oral testimony at a hearing or at trial consistent with the statements made in this declaration.

12. I reserve the right to supplement opinions rendered in this declaration as a result of the testimony and opinions of other witnesses or other information which might exist and which may be presented during the remainder of discovery or during trial of this matter, including graphic or demonstrative materials not yet prepared.

13. I declare under penalty of perjury under the laws of the United States that the foregoing is true and correct to the best of my knowledge and belief.

DATED: Philadelphia, Pennsylvania
March 19, 2007

/s/ Thomas R. Kadesch
Thomas R. Kadesch, Ph.D.

Index of Exhibits	
Exhibit 1	Curriculum Vitae for Thomas Robert Kadesch.
Exhibit 2	U.S. Patent No. 4,703,008, and the claims for U.S. Patent Nos. 5,547,933; 5,441,868; 5,618,698; 5,756,349; 5,955,422 and 5,621,080.
Exhibit 3	*Biochemistry* (Lubert Stryer 2d ed. 1981) (1975).
Exhibit 4	Por-Hsiung Lai et al., *Structural Characterization of Human Erythropoietin*, 261 THE J. OF BIO. CHEM. (ISSUE 7) 3116 (1986).
Exhibit 5	Michael Recny et al., *Structural Characterization of Natural Human Urinary and Recombinant DNA-derived Erythropoietin*, 262 THE J. OF BIO. CHEM. (ISSUE 35) 17156 (1987).

EXHIBIT 4

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Structural Characterization of Human Erythropoietin*

(Received for publication, August 26, 1985)

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Erythropoietin is the primary regulator of red blood cell formation in mammals. Because of its extreme scarcity, very little information is available regarding structural features of this important glycoprotein. We report here the primary structure of human urinary erythropoietin, determined by protein sequencing. In addition, the sites of glycosylation, assignment of disulfide bonds, and the circular dichroism of the hormone analyzed for secondary structure in comparison with the prediction from the sequence are presented.

Although the existence of a humoral factor regulating red blood cell formation was postulated as early as 1906 (1), very little information about the structure of erythropoietin (EPO[1]) has been published because of its very limited availability. The hormone derived from plasma of anemic sheep was purified in 1971 (2), but too little was obtained to learn more than its apparent molecular weight (3), amino acid composition, and the fact that it was glycosylated (4). Human EPO purified from the urine of patients with aplastic anemia (5) consists of two distinguishable forms with differing content of carbohydrate; one termed α containing 31% and one termed β with 24% carbohydrate. All of the carbohydrate appears to be *N*-linked (6). The apparent M_r was estimated to be 34,000 and some information about its domain structure has been published (7).

MATERIALS AND METHODS AND RESULTS[2]

DISCUSSION

Primary Structure—The complete amino acid sequence for the human EPO protein (565 μg of EPO used) is shown in Fig. 1. The sequenced region of the intact protein and the various fragments used to establish the order of sequences obtained by cleavage of the protein are also indicated. As shown in Fig. 1, 77 residues of sequence information could be obtained with only 100 μg of protein by using the technique of *in situ* CNBr cleavage. This sequencing technique should have application in the structural analysis of proteins which are only available in minute quantities. Proteins containing more than 1 methionine residue can also be analyzed by this technique if primary amine-specific reagents such as fluorescamine or *o*-phthalaldehyde are used to strategically block unwanted peptides in the CNBr peptide mixture at a point where a proline residue is at the exposed NH_2 terminus (8).

The amino acid sequence of human EPO, shown in Fig. 1, contains 166 residues and has a calculated M_r = 18,398 for the protein moiety. It contains three more basic amino acids than acidic ones. Charged residues constitute 27% of the total and are irregularly distributed, except that no charged residues occur in region 77–88 and both the NH_2- and C-terminal ends are relatively highly charged. It is interesting to note that although glycine and proline residues which are known to be strong breakers of α-helix and β-sheet structures, are randomly distributed through most of the molecule, no such residues occur in regions 4–27 and 130–150. A high degree of α-helix structures may be possible in these regions.

In a previous report Yanagawa *et al.* (9) published the sequence of the 30 NH_2-terminal residues of human urinary EPO purified by an immunoaffinity method. This sequence differs from the one in this paper at residues 5 (Leu instead of Lys) and 14 (Arg instead of Ile); residues 3 and 24 were not specified; we find Pro at position 3, and assigned Asn for position 24. In our studies, this region of the EPO molecule has been sequenced four times using intact protein as well as tryptic and *Staphylococcus aureus* V8 protease peptides. In addition, our data for these positions are confirmed by the DNA sequence of the human gene (10, 11).

Glycosylation Sites—The sequence we report has three possible glycosylation sites at positions 24, 38, and 83, according to the presence of Asn-X-Ser/Thr (12). The assignment of Asn at these positions was also supported by the evidence that amino acid composition analysis of peptide T30 and T38 indicated the presence of glucosamine the *N*-acetylated species of which is the linking sugar on the asparagine residue (data not shown). Sequence analysis of peptides T28 and 2S63 indicated a serine at position 120 and no identifiable PTH for position 126. However, amino acid composition analysis revealed the presence of 2 serine residues in this fragment. Analysis of the DNA sequence indicated that a serine is present at position 126 (10, 11). One possible explanation for these results is that position 126 is a glycosylated serine. In fact, our preliminary results indicated that galactosamine whose precursor, *N*-acetylgalactosamine, is the linking sugar at hydroxy amino acids was detected in the composition analysis of peptides T28 and 2S63 (data not shown).

* This work was supported in part by Grant HL 21676 from the National Heart, Lung and Blood Institute, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: EPO, erythropoietin; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoins; PTC, phenylthiocarbamyl; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

[2] Portions of this paper (including "Materials and Methods," "Results," Figs. 2 and 3, Tables 1 and 2, Footnote 3, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2899, cite the authors, and include a check or money order for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

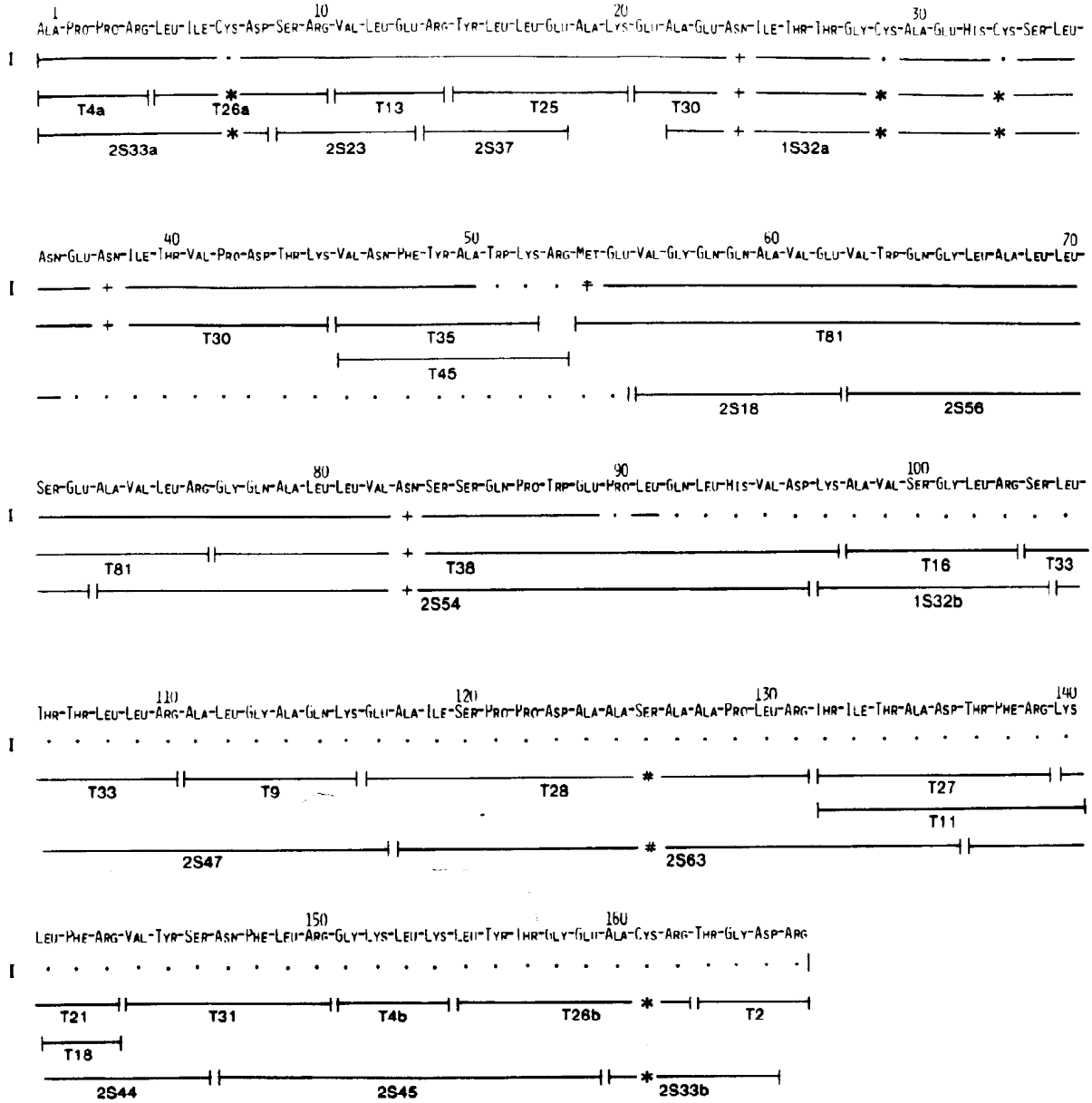

FIG. 1. **Amino acid sequence analysis of human erythropoietin.** Sequences analyzed with intact urinary EPO and peptides derived from urinary protein are indicated by *solid lines* under the residues comprising the protein or the peptide. The *letter code* indicates source of peptides and the cleavage method used to produce the peptides: *I*, intact protein; *T*, trypsin; *S*, *S. aureus* V8 protease; *1S*, digest C; *2S*, digest D. Results for sequence analysis with intact protein represents data obtained from two separate determinations. The number of the peptide identifies it in the respective HPLC chromatogram. The *solid line* indicates the results of automated Edman degradation. *Dots on the line* indicate respective residues which are not identified by automated Edman degradation. The letter *a* or *b* behind a peptide number indicates the pair of peptides which were co-isolated from peptide maps. * indicates identification of cysteine residues: Cys 7 and Cys 161 form one cystine and Cys 29 and Cys 33 form another cystine. The + indicates glycosylated asparagine. The # indicates a tentatively assigned glycosylated serine. The ‡ indicates identification of the methionine residue on the basis of cleavage chemistry and sequence analysis of T81.

Determination of Disulfide Bonds—As shown in Fig. 1, EPO contains 4 cysteine residues. Although during sequence analysis no PTH-cysteine could be detected for these 4 residues, we have assigned cysteine residue for positions 7, 29, 33, and 161 based on the following observations.

1. Two pairs of peptides, T26a/T26b and 2S33a/2S33b, copurified with equal recovery from high performance liquid chromatography of digests A and D, respectively. 2. PTH-cystine (eluted between PTH-threonine and PTH-glutamine under the described analytical conditions) could be detected at the seventh step of Edman degradation of both peptide pairs T26a/T26b and 2S33a/2S33b (Table 2), when degradation products were promptly analyzed. 3. PTH-cystine was detected at the thirteenth step of Edman degradation of