

Molecular principles of drug design

Development of biologic therapeutics.

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Biologics or biological therapeutics

- officially (WHO) „biological and biotechnological substances“

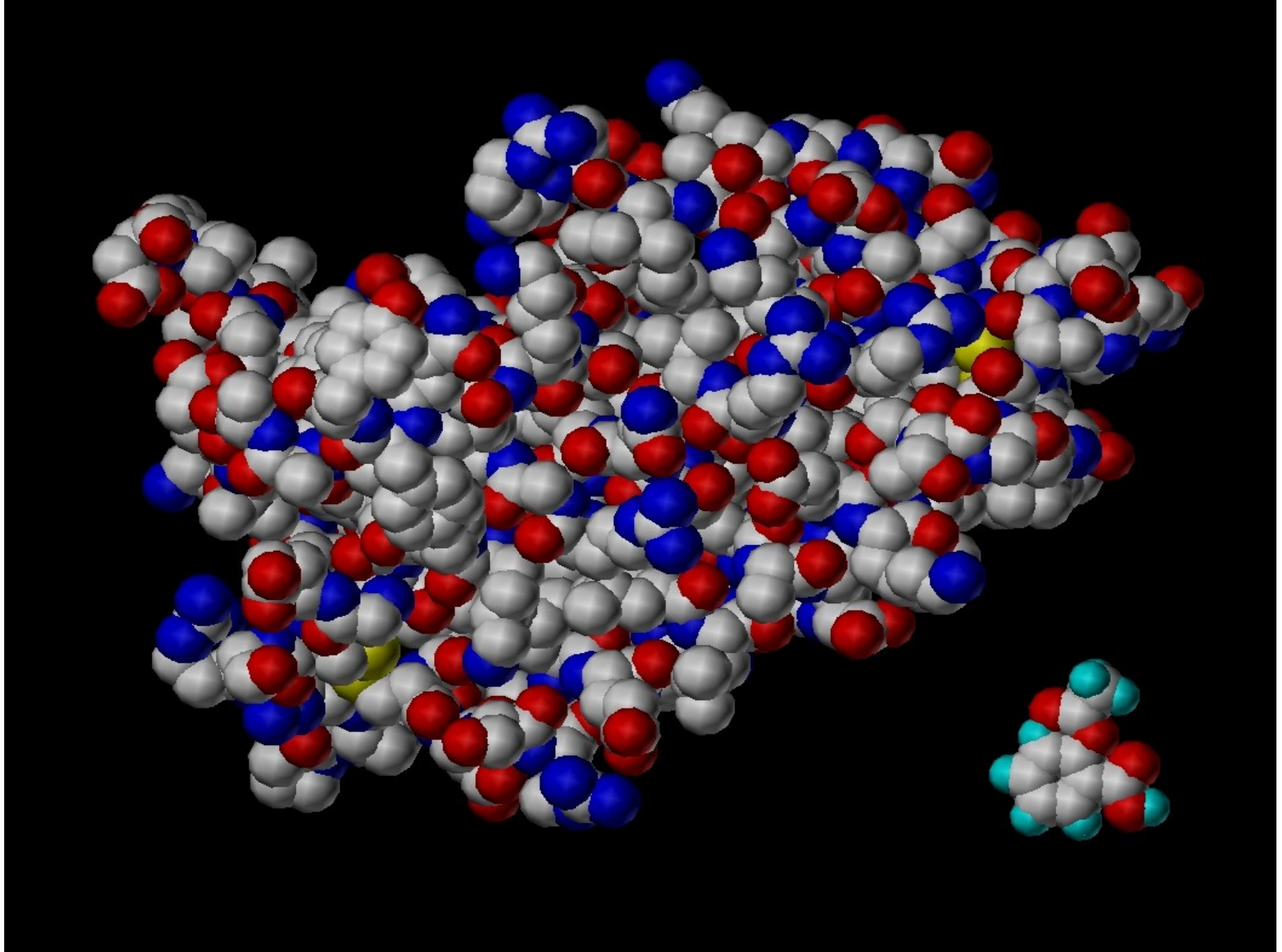
Basic characterisation of biologics

- typically obtained by other way than by classical chemical synthesis (semisynthetic modifications are possible)
- typically $M_r > 1000$ (up to 1000 „small molecules“) - greater, more complex, usually exhibit a *primary structure* (a sequence of amino acids or nucleotides), a *secondary structure* (α -helix, “folded sheet”, influence of -S-S- bridges), a *tertiary structure* (general space arrangement of a monomeric molecule) and a *quarternary structure* (grouping of monomers); many proteins are glycosylated
- but both above conditions need not be necessarily fulfilled for classification of a drug as a biologic

Differences in production of “small molecules” and biologics

- small molecules – classical organic synthesis: chemicals with exactly defined chemical structure and purity react under exactly defined conditions with a predictable and precisely verifiable results
- biologics- preparation by „harvesting“ of compounds produced and secreted by artificially constructed cells (genetic engineering)

An illustration of the difference between a biologic and a “small molecule”
erythropoietin and acetylsalicylic acid

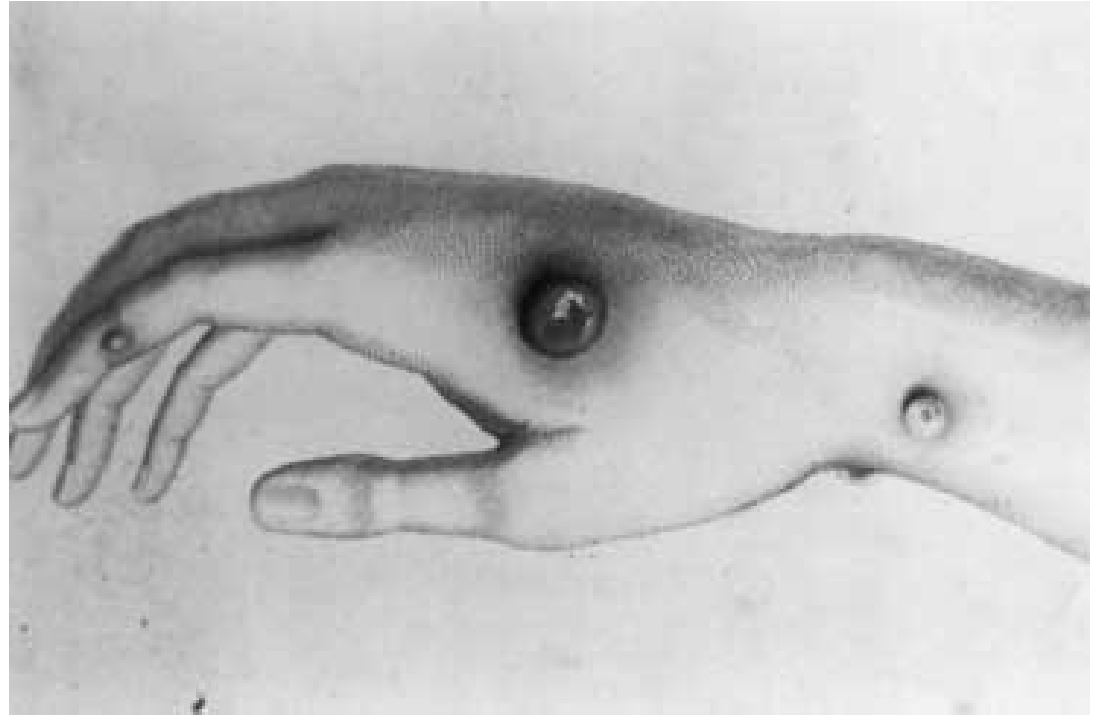


History of biologic therapeutics

- the Antiquity and the Middle Ages: usage of leeches for treatment of circulation and blood disorders (hirudin)
- classical vaccines: preparation of dead or attenuated bacterial cultures or attenuated or inactivated viruses (e.g. pox: transfer of the infection „from a skin to a skin“ has been known long since around 1000 A.D. in China; 1796 – Edward Jenner demonstrated that putting of the purulence from a furuncle of the cowpox in under the skin protected against the infection with pox; 1805 – 1st vaccine against pox was prepared on the calf skin in Italy; 1864 – mass usage of this vaccine; after 1940 – lyophilized vaccines (Collier))



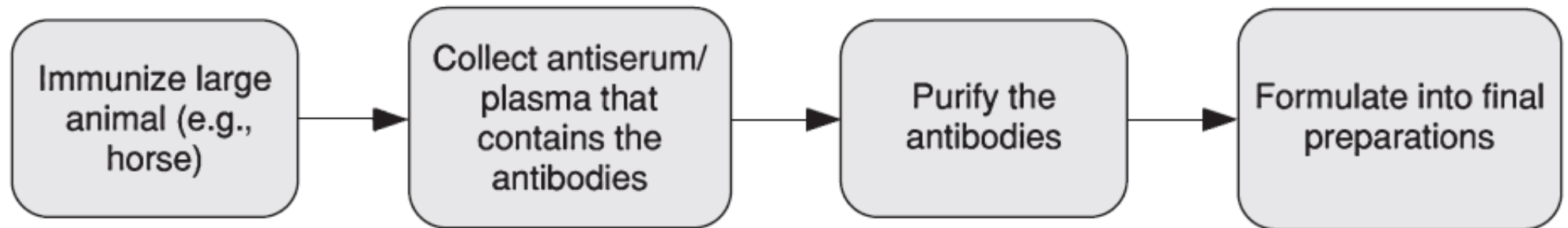
Edward Jenner. Lithograph based on the painting by J.R. Smith (1801).
Courtesy of the Institute of the History of Medicine, The John Hopkins University, where the original painting is on display.



The cowpox lesion on the hand of the milkmaid, Sarah Nelmes, from which Jenner took pustular material to inoculate the boy James Phipps, May 1796. Case XVI of Edward Jenner's 1798 report on vaccination (2).

History of biologic therapeutics - continued

- (poly-clonal) antibodies („sera“) - immunisation of a suitable production macro-organism (eg. horse, rabbit) with a noxious agent (a toxin, e.g. a snake poison), a serum acquired from the blood used as an antidote; monoclonal antibodies for analytic and diagnostic purposes, then a suitable transformation (RIA, ELISA)
- peptides – isolation from biological material (insulin: Banting and Best 1921)

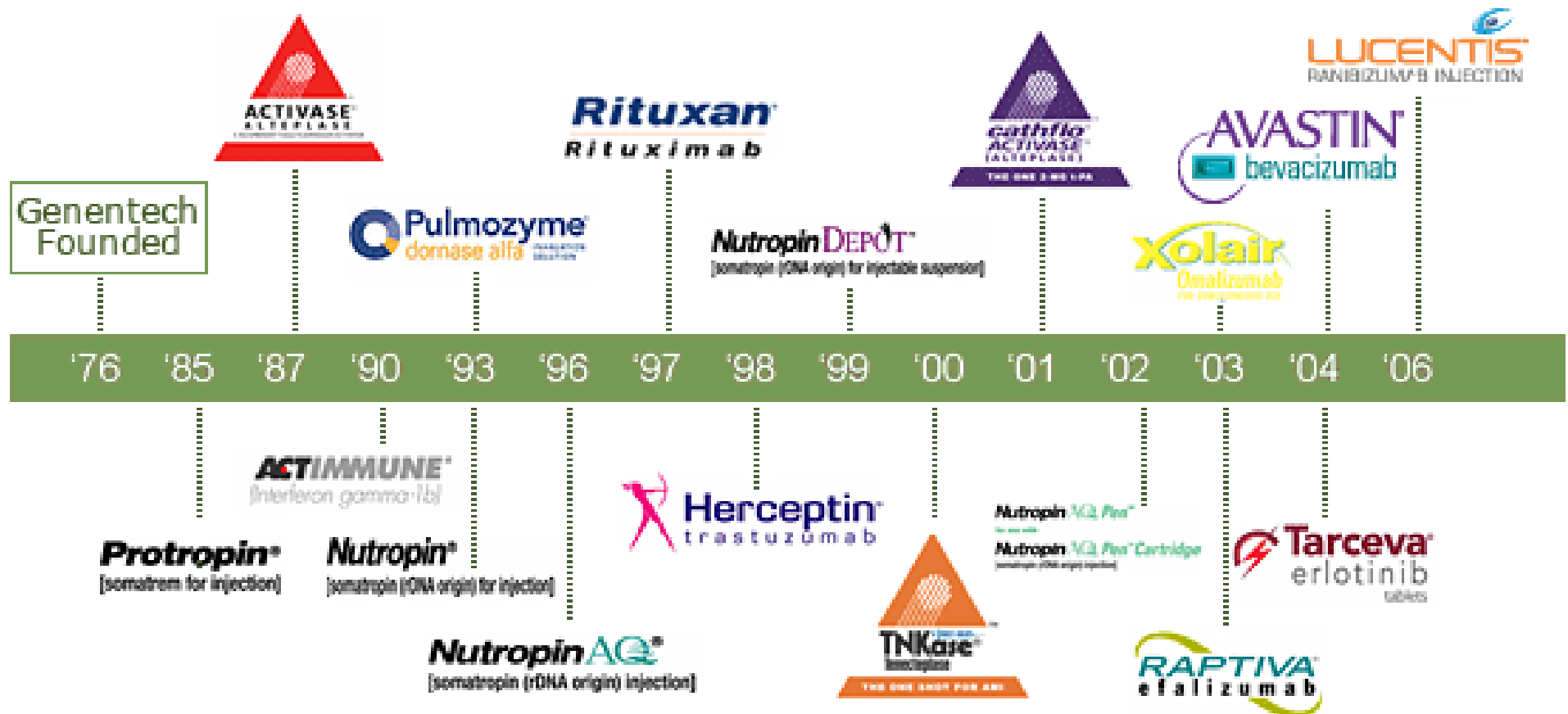


Production of polyclonal antibodies from horse antisera.

More recent history of biologic therapeutics – genetic engineering

- 1977 - somatostatin first time prepared by a recombinant technology in *E. coli* (Genetech, USA)
- 1978 – human insulin cloned
- 1982 – recombinant human insulin prepared in *E. coli* marketed
- 1984 – Factor VIII of the blood clotting first time prepared in a laboratory
- 1985 – FDA approved somatrem, a somatotropin analogue
- since 1980th – development of therapeutic monoclonal antibodies (mabs)
- in 1980th 80 % of mabs of mouse origin, in 2000th only 7 %
- OKT3 - murine anti-CD3 antibody was the first mab approved by FDA for treatment of organ transplant rejection

History of biologic therapeutics from the point of view of a corporation (Genentech)



Development and authorisation of new biologic therapeutics

EMA (EU): normal approval procedure like for any other drug; most biotherapeutics in EU have been approved by the centralized procedure (i.e. one approval procedure for all EU countries)

FDA (USA): possibility of taking part into so called Fast Track Drug Development Program (since 1998, revised 2004) – the prerequisite is the usefulness for serious or life endangering condition and legitimated hope for better clinical efficacy than up to the present time used drugs

When the patent of original drug expires: generics and „biosimilars“

Generics: small molecules – contain the same active compound as the original and reach 80 – 105 % of the bioavailability of the original

„Biosimilar“ (EMA) = „follow-on protein“ = „similar biotherapeutic product (WHO) = „similar biologic“ (India) etc. - a biotherapeutic product that is similar in terms of quality, safety and efficacy to an already licensed reference biotherapeutic product. (= “originator product”) - [WHO definition]

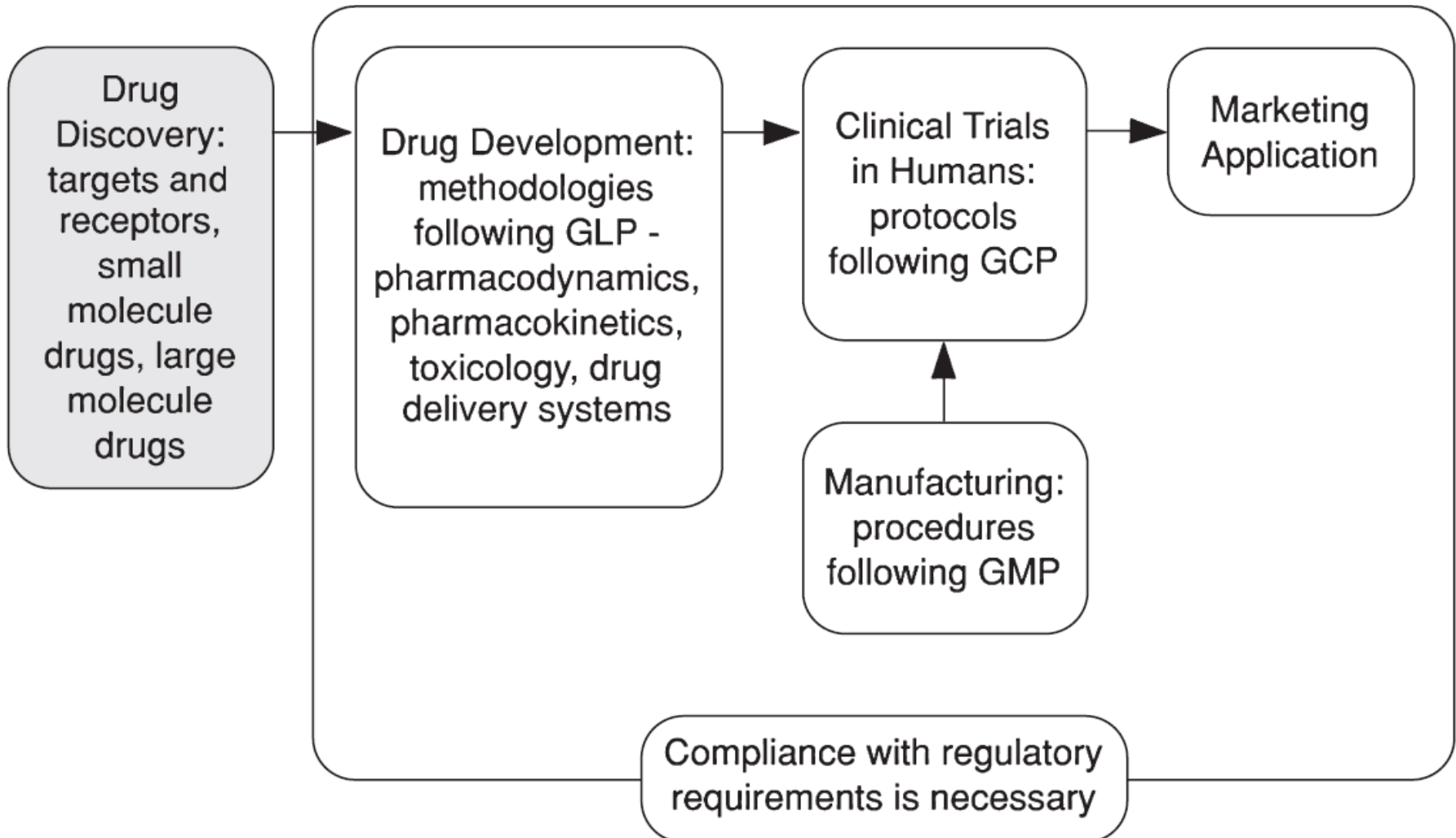
Different approaches of FDA (USA) and EMA (EU) to biosimilars´ approvals

- EMA: approval procedure for biosimilars exists and approvals successfully has been running 2007
- FDA: all backgrounds for such a procedure had been ready for many years, the approvals have started in 2015
- Common „biosimilars cluster“ of FDA and EMA, later joined also Health Canada and Pharmaceuticals and Medical Devices Agency (PMDA - Japan)

Pharmacological classification of biological and biotechnological substances in after WHO

- Drugs for alimentary tract and metabolism: insulins.
- Anti-infectives: antimicrobial, bactericidal permeability increasing polypeptides, human papillomavirus.
- Antineoplastics: peptide vaccines, recombinant vaccines, toxins.
- Blood and agents acting on the haemopoietic system: antithrombins, blood coagulation cascade inhibitors, blood coagulation factors, erythropoietin type blood factors, heparin derivatives including low molecular mass heparins, heparinoids, hirudin derivatives, trombosmodulins.
- Immunomodulators and immunostimulants: colony stimulating factors, interferons, interleukin receptor antagonists, interleukin type substances, monoclonal antibodies, receptor molecules, native or modified, tumor necrosis factor antagonists.
- Hormones, hormone antagonists, hormone-release stimulating peptides or hormone-release inhibiting peptides (excluding insulins): growth hormone (GH) derivatives, its antagonists, oxytocin derivatives, pituitary / placental glycoprotein hormones, pituitary hormone-release stimulating peptides, synthetic polypeptides with corticotropine-like action, vasoconstrictors, vasopressin derivatives.
- Various: „antisense“ oligonucleotides, enzymes, gene therapy products, growth factors, peptides and glycopeptides not classified above.

Examples of development in a particular groups of biologic therapeutic



Erythropoietins

APPRL I CDSR	VLERYLLEAK	EAEN I TTGCA
EHCSLNEN I T	VPDTKVN FYA	WKRMEV GQQA
VEVWQGLALL	SEAVLRGQAL	LVNSSQPWEP
LQLHVDKAVS	GLRSLTTLLR	ALGAQKEA I S
PPDAASAAPL	RT I TADTFRK	LFRVYSNFLR
GKLKLYTGEA	CRTGD	

M_r about 30 600

CAS 113427-24-0

erythropoietin (EPO)

= glycosylated protein from 165 AA

M_r of protein aglycon 18235.87

Erythropoietini solutio concentrata EP

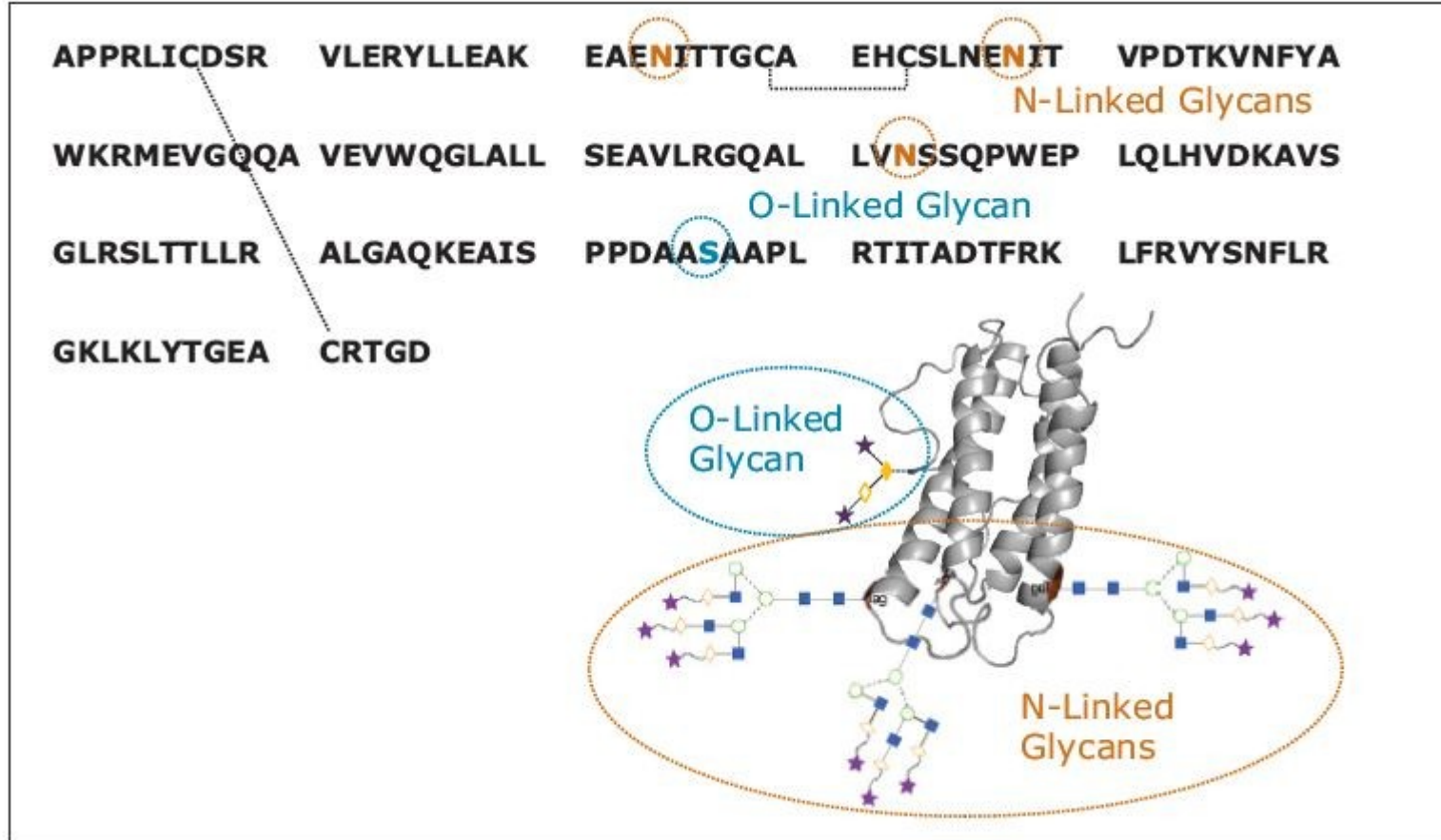
= a solution containing a group of closely related glycoproteins, which are not to distinguish from the natural human erythropoietin (human urine erythropoietin, huEPO), from the point of view of 165 amino acids sequence and their average profile of glycosylation

- naturally released from kidneys of adults and in liver of foetus
- stimulates stem cells of bone marrow to proliferation and differentiation
- produced *in vitro* mostly in rodent cell lines by a method based on the recombinant DNA technology
- **INN names: epoetin + greek letter spelt in full** (eg. epoetin beta)
- various epoetins differ in glycosylation, complex branched oligomeric sugar chains are attached
- treatment of anaemia in chronic kidney failure, missused for doping

A brief overview of epoetins' development

INN name: epoetin	Year of discovery /approval	Production organism / tissue	M _r CAS	Glycosylation pattern	Originator product/biosimilar	Brand names ®, generic codes
alfa	2000	Chinese hamster ovary	113427-24-0	similar to uhEPO	orig/biosim	Eprex , Binocrit, Abseamed
beta	1997	Chinese hamster ovary	122312-54-3		orig	Neorecormon
gama	1990	C127 murine cells transfected with huEPO cRNA	28 000-31 000 130455-76-4		orig	TYB-5220
delta	2002 - 2009	human fibrosarcoma cell line HT-1080	261356-80-3	less <i>O</i> -acetyls in <i>O</i> -glycan chains; similar to uhEPO	orig	Dynepo
epsilon	1995		154725-65-2		orig	
zeta	2007	Chinese hamster ovary	32 000-40 000 604802-70-2		biosim. of EPO alfa	Silapo, Retacrit
theta	2009	Chinese hamster ovary	762263-14-9	sugars represent 40 % of total M _r	orig	Biopoin, Eporatio
kappa	2010	Chinese hamster ovary	11096-26-7		biosim. of EPO alfa	Epoetin alfa BS injection ®
omega	1986	BHK-21 cells of Chinese hamster kidney	148363-16-0	greater sialylation of tetraantenary <i>N</i> -linked chains	orig	Epomax, Hemax

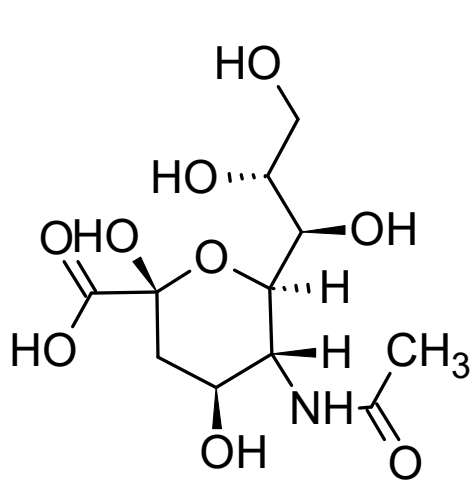
Epoetins' glycosylation



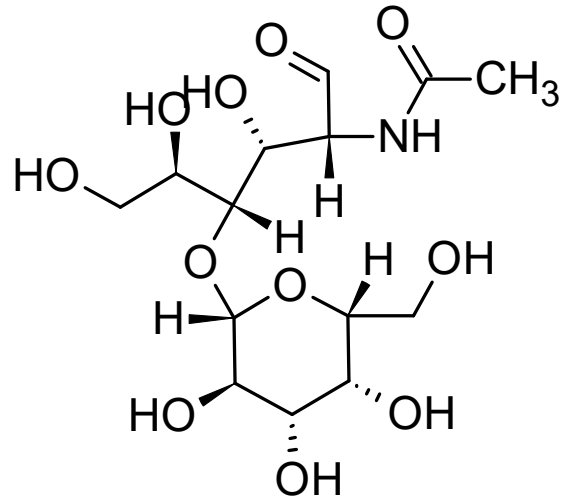
Sites of *N*-glycosylation: Asn24, Asn38, Asn83 (= N24, N38, N83)

Site of *O*-glycosylation: Ser126 (= S126)

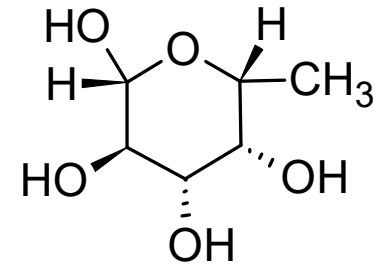
Epoetins' glycosylation: some more specific occurring sugars



N-acetylneuraminic acid (= sialic acid)



N-acetyllactosamine



L-fucose

Epoetins' glycosylation: secondary structure of N-attached oligosaccharide chains

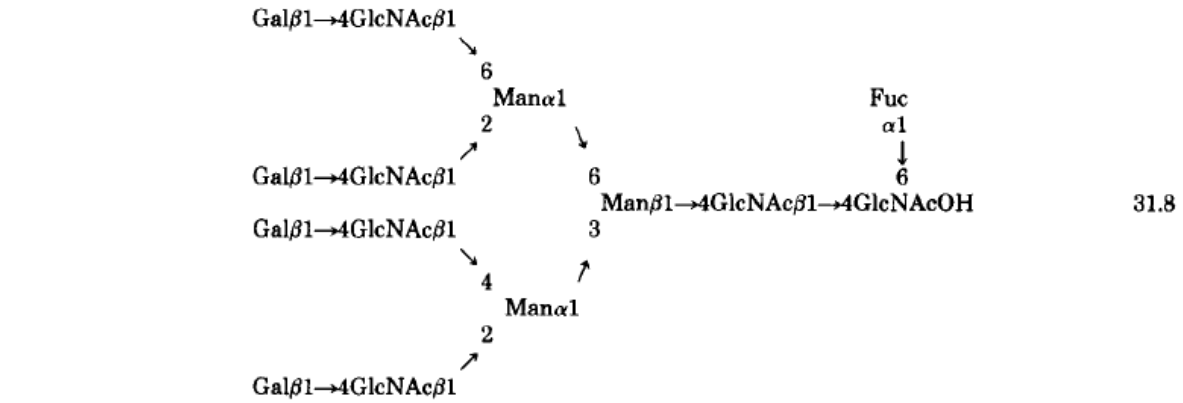
Structures of asialo N-linked saccharides obtained from recombinant erythropoietin

Carbohydrate Structure of Human Recombinant Erythropoietin

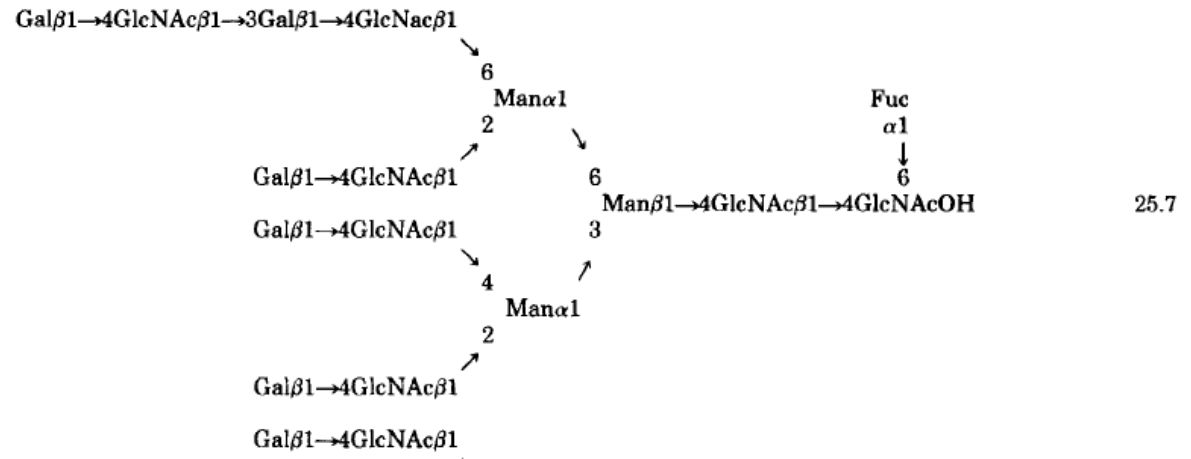
Saccharide	Structure ^e	Relative amount %
Biantennary	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \\ \searrow 6 \\ \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcOH} \\ \nearrow 3 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \end{array}$	1.4
	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \\ \searrow 6 \\ \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcOH} \\ \nearrow 3 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \\ \searrow 4 \\ \text{Man}\alpha 1 \\ \nearrow 2 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \end{array}$	3.5
Triantennary	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \\ \searrow 6 \\ \text{Man}\alpha 1 \\ \searrow 6 \\ \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcOH} \\ \nearrow 3 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \end{array}$	6.5
	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \\ \searrow 6 \\ \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 \text{GlcNAcOH} \\ \nearrow 3 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 3 \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \\ \searrow 4 \\ \text{Man}\alpha 1 \\ \nearrow 2 \\ \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \end{array}$	3.5

Epoetins' glycosylation: secondary structure of *N*-attached oligosaccharide chains continued

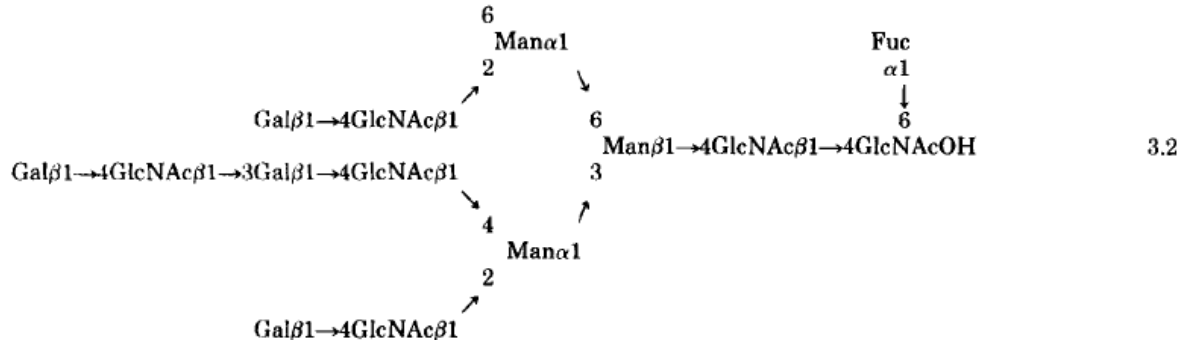
Tetraantennary



Tetraantennary Lac₁



Tetraantennary Lac₁

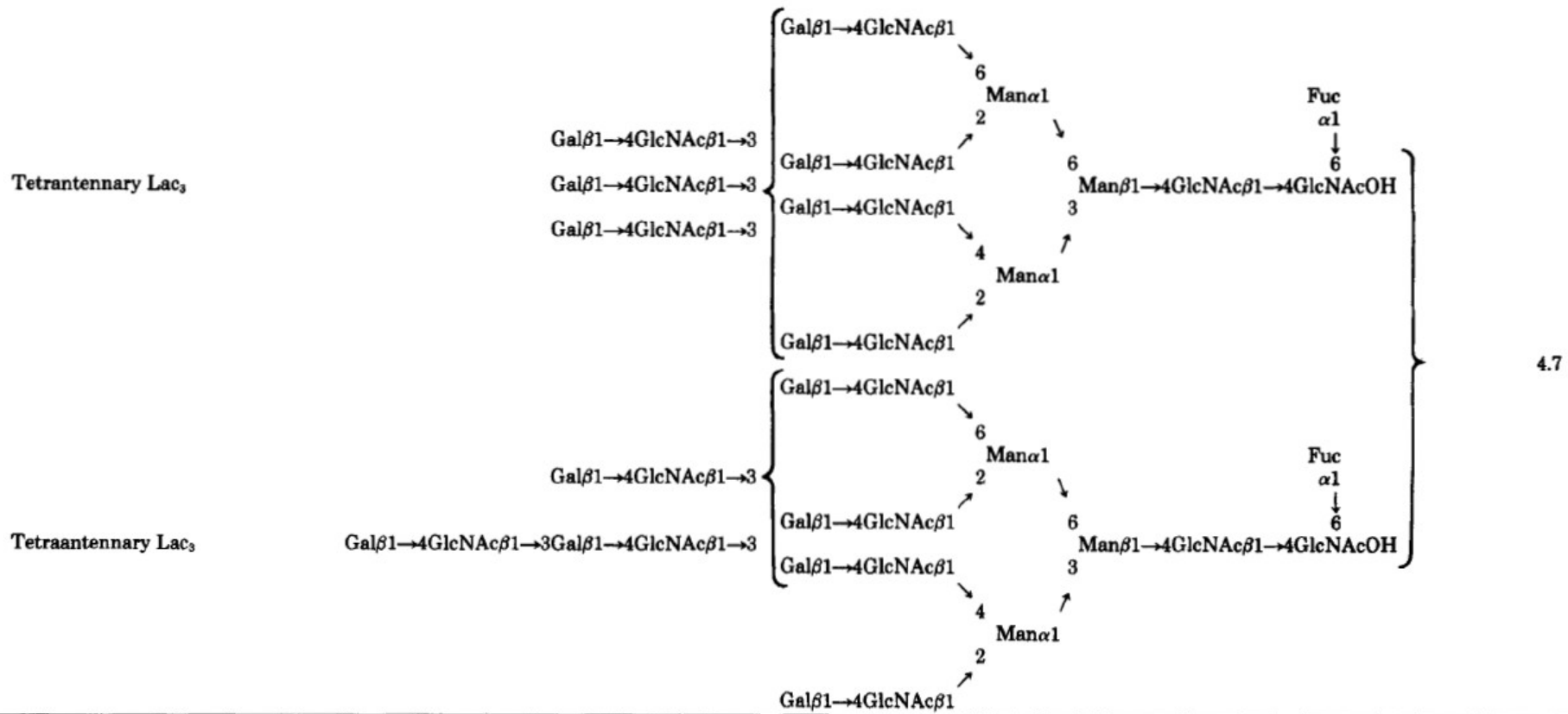


Epoetins' glycosylation: secondary structure of *N*-attached oligosaccharide chains continued

TABLE III-Continued

Saccharide	Structure ^a	Relative amount %
Tetraantennary Lac ₁		3.2
Tetraantennary Lac ₂		13.2
Tetraantennary Lac ₂		3.3

Epoetins' glycosylation: secondary structure of *N*-attached oligosaccharide chains continued



* 15% of the saccharides lack fucose attached to the reducing terminal *N*-acetylglucosamine.

Differences in individual epotins' glycosylation pattern: CZE in accordance with the European Pharmacopoea

B. Capillary zone electrophoresis (2.2.47).

Test solution. Dilute the preparation to be examined with water R to obtain a concentration of 1 mg/mL. Desalt 0.25 mL of the solution by passage through a micro-concentrator cartridge provided with a membrane with a molecular mass cut-off of not more than 10 000 Da. Add 0.2 mL of water R to the sample and desalt again. Repeat the desalting procedure once more. Dilute the sample with water R, determine its protein concentration as described under Tests and adjust to a concentration of approximately 1 mg/mL with water R.

Reference solution. Dissolve the contents of a vial of erythropoietin for physicochemical tests CRS in 0.10 mL of water R. Proceed with desalting as described for the test solution.

Capillary:

- material: uncoated fused silica;
- size: effective length = about 100 cm, Ø = 50 µm.

Temperature: 35 °C.

CZE buffer concentrate (0.1 M sodium chloride, 0.1 M tricine, 0.1 M sodium acetate). Dissolve 0.584 g of sodium chloride R, 1.792 g of tricine R and 0.820 g of anhydrous sodium acetate R in water R and dilute to 100.0 mL with the same solvent.

1 M putrescine solution. Dissolve 0.882 g of putrescine R in 10 mL of water R. Distribute in 0.5 mL aliquots.

CZE buffer (0.01 M tricine, 0.01 M sodium chloride, 0.01 M sodium acetate, 7 M urea, 2.5 mM putrescine). Dissolve 21.0 g of urea R in 25 mL of water R by warming in a water-bath at 30 °C. Add 5.0 mL of CZE buffer concentrate and 125 µL of 1 M putrescine solution. Dilute to 50.0 mL with water R. Using dilute acetic acid R, adjust to pH 5.55 at 30 °C and filter through a membrane filter (nominal pore size 0.45 µm).

Detection: spectrophotometer at 214 nm.

Set the autosampler to store the samples at 4 °C during analysis.

Preconditioning of the capillary: rinse the capillary for 60 min with 0.1 M sodium hydroxide filtered through a membrane filter (nominal pore size 0.45 µm) and for 60 min with CZE buffer. Apply voltage for 12 h (20 kV).

Differences in individual epotins' glycosylation pattern: CZE in accordance with the European Pharmacopoea continued

Between-run rinsing: rinse the capillary for 10 min with [water R](#), for 5 min with [0.1 M sodium hydroxide](#) filtered through a membrane filter (nominal pore size 0.45 µm) and for 10 min with CZE buffer.

Injection: under pressure or vacuum.

Migration: apply a field strength of 143 V/cm (15.4 kV for capillaries of 107 cm total length) for **80 min**, using CZE buffer as the electrolyte in both buffer reservoirs.

System suitability: in the electropherogram obtained with the reference solution, a pattern of well separated peaks corresponding to the peaks in the electropherogram of erythropoietin supplied with [erythropoietin for physicochemical tests CRS](#) is seen, and the largest peak is at least 50 times greater than the baseline noise. If necessary, adjust the sample load to give peaks of sufficient height. Identify the peaks due to isoforms 1 to 8. Isoform 1 may not be visible. The peak due to isoform 8 is detected and the resolution between the peaks due to isoforms 5 and 6 is not less than 1. Repeat the separation at least 3 times. The baseline is stable, showing little drift, and the distribution of peaks is qualitatively and quantitatively similar to the distribution of peaks in the electropherogram of erythropoietin supplied with [erythropoietin for physicochemical tests CRS](#). The relative standard deviation of the migration time of the peak due to isoform 2 is less than 2 per cent.

Limits: identify the peaks due to isoforms 1 to 8 in the electropherogram obtained with the test solution by comparison with the electropherogram obtained with the reference solution. Calculate the percentage content of each isoform from the corresponding peak area. The percentages are within the following ranges:

Isoform Content (per cent)

1 0 - 15

2 0 - 15

3 1 - 20

4 10 - 35

5 15 - 40

6 10 - 35

7 5 - 25

8 0 - 15

Differences in individual epotins' glycosylation pattern: CZE in accordance with the European Pharmacopoea continued

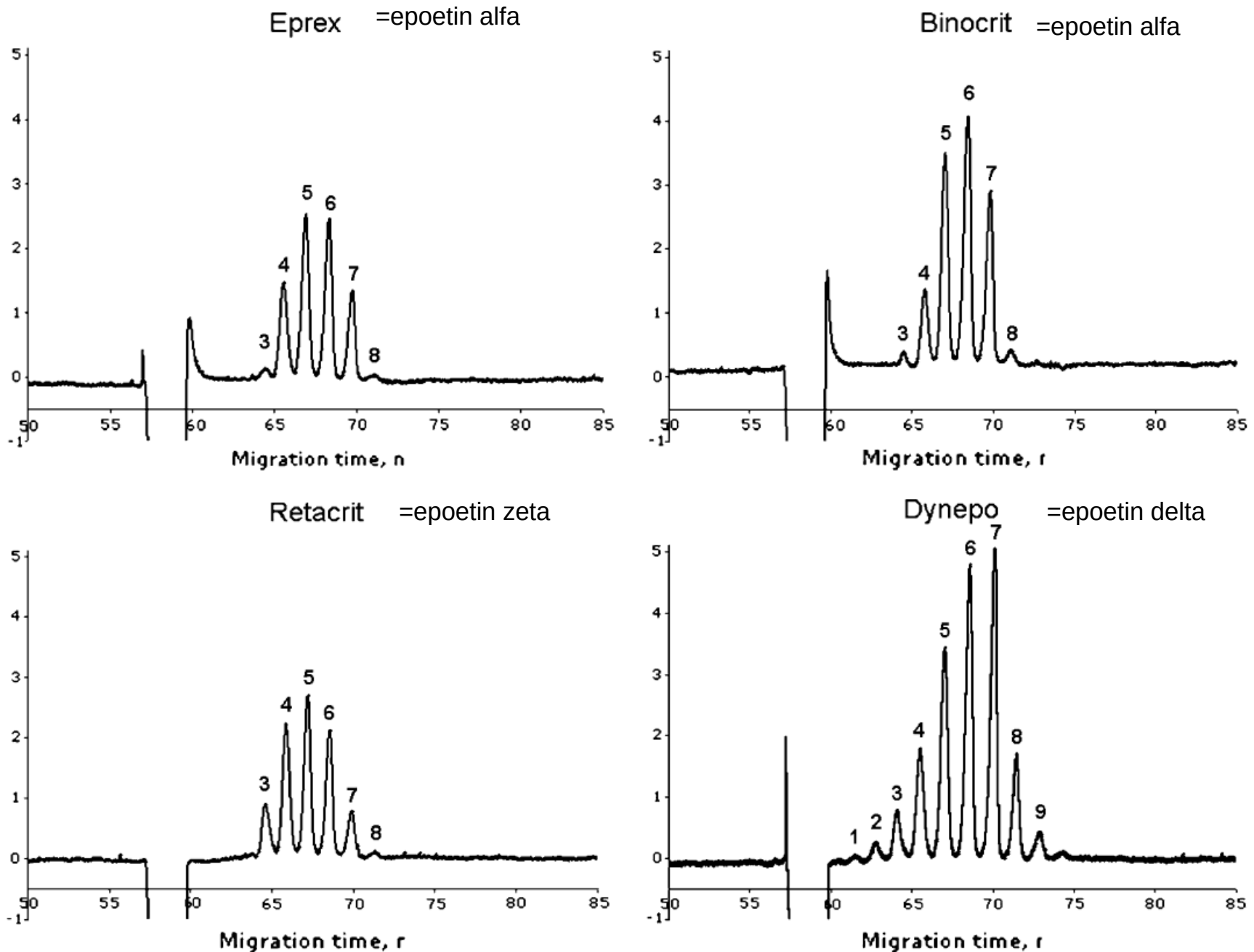
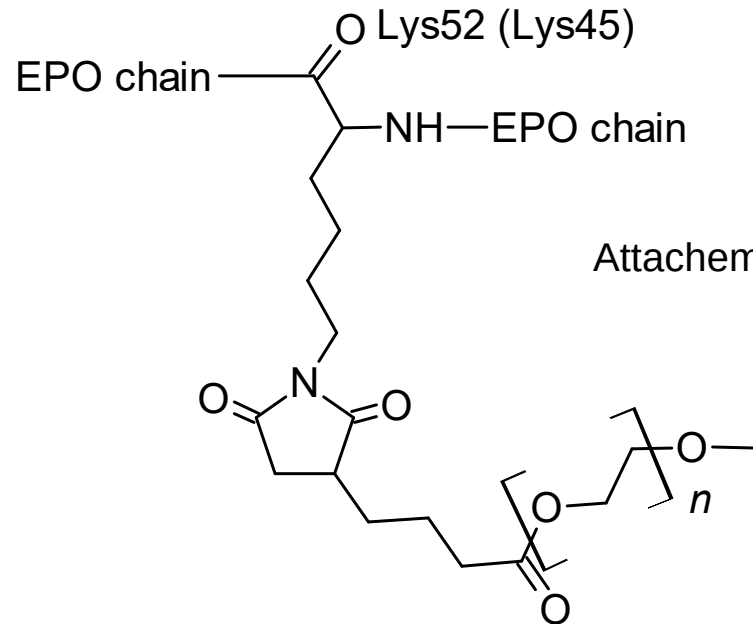


Fig. 3 CE-UV analysis of the four EPO products.

Epoetin conjugates
Methoxy-polythylenglycol-epoetin beta

Total M_r cca 60 000



Attachement of poly(oxoethylene) chain to EPO by a „monamide“ linker

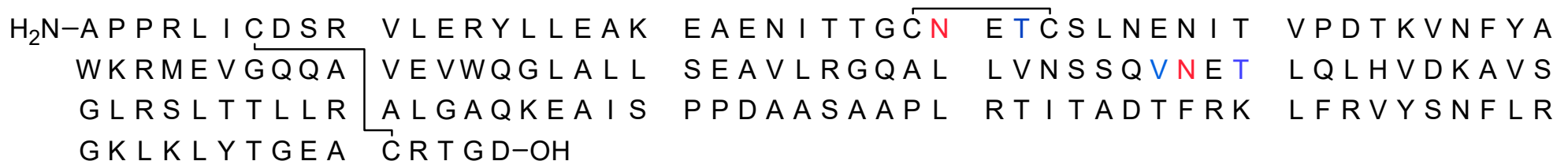
Plasmatic $T_{1/2}$ cca 139 h \Rightarrow „continuous erythropoietin receptor activator“, CERA
Mircera [®] (s.c. or i.v.) for treatment of anemia in chronic renal disease

Epoetin analogues with altered protein sequence

Darbepoetin alfa

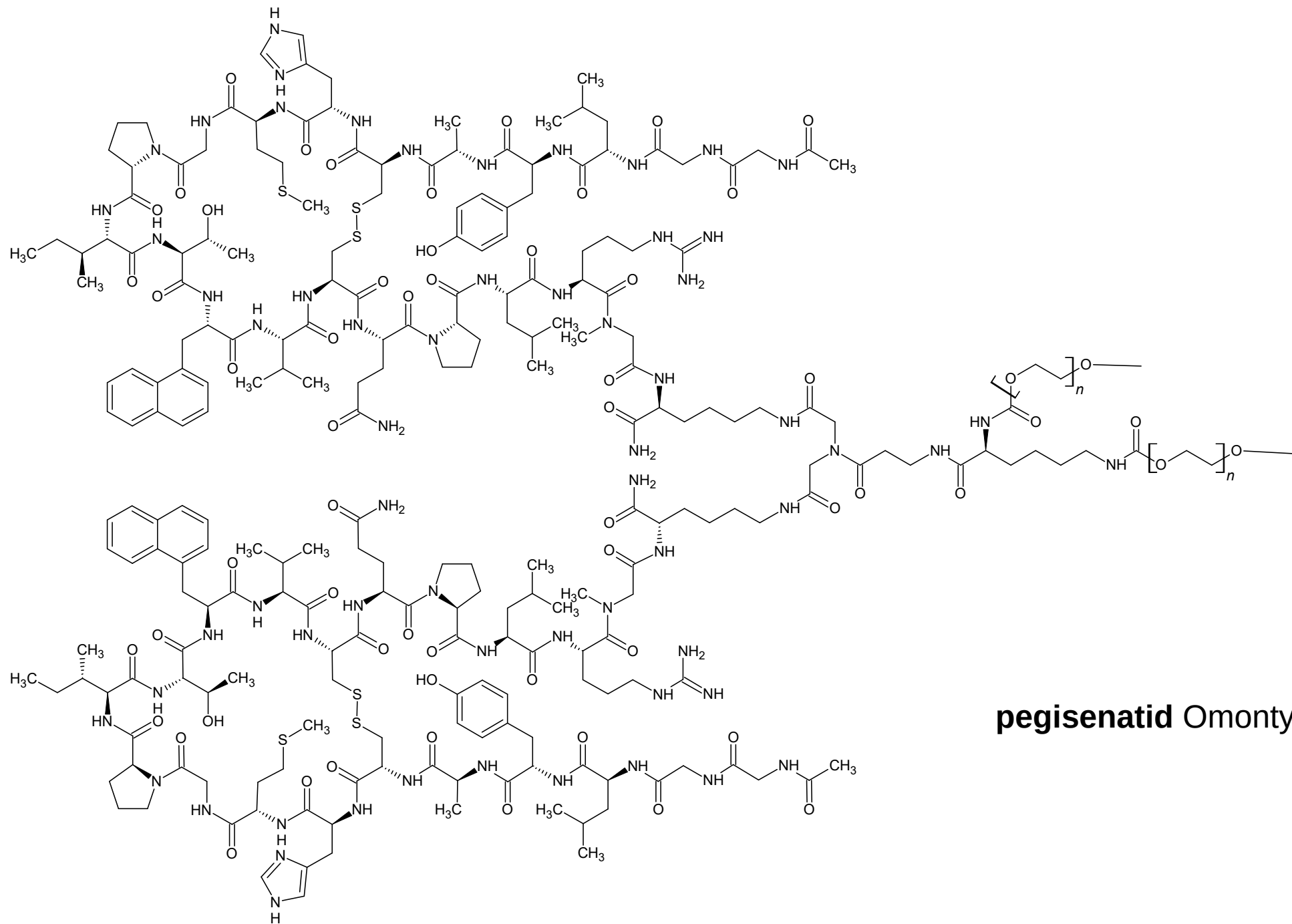
- sequence of EPO alfa changed: Asn30, Thr32, Val87, Asn88 and Thr90 \Rightarrow 2 new sites of *N*-glycosylation \Rightarrow 5 sites of *N*-glycosylation in total; 2 new oligosaccharide chains attached
- total M_r 30 000 – 37 000
- recombinant
- indicated for treatment of anemia caused by a chemotherapy of non-myeloid cancers or by chronic renal failure

Aranesp[®] (originator); Nespo[®] (biosimilar – approved in EU 2001 - 2008)



Primary structure of darbepoetin alfa aglycone. New asparagine residues, to which new carbohydrate chains are attached, are in red, other changed amino acid residues in blue.

EPO receptor agonists structurally unrelated to EPO



pegisenatid Omontys®

Pegisenatid: a blind path

- non-glycosylated covalent dimer consisting of 2 equal peptide chains linked by ϵ -amino group of C-terminal Lys to both carboxyl of iminediacetic acid
- this is acylated with the fragment of 4-aminobutyric acid which is then acylated with N^1, N^6 -dicarboxyLys
- its carboxyls are then esterified with ω -methoxy-poly(oxoethylene) chains
- peptide chains are cyclized by S-S bridges, each contains also a fragment of naphthalen-1-ylalanine
- M_r of PEG chains 40 000
- M_r of peptide part 5 714
- shortly used in USA for treatment of anemia of patients with symptomatic renal disease undergoing dialysis but withdrawn due to increased risk of death for cardiovascular causes and for dangerous allergies; the approval procedure in EU then stopped