Supporting Information for

**Stabilization of bacterially expressed Erythropoietin by single site-specific introduction of short branched PEG chains at naturally occurring glycosylation sites**

Eugenia Hoffmann,\textsuperscript{a} Katharina Streichert,\textsuperscript{a} Nicole Nischan,\textsuperscript{b} Carina Seitz,\textsuperscript{c} Thomas Brunner,\textsuperscript{c} Sergei Schwagerus,\textsuperscript{b} Christian Hackenberger\textsuperscript{b,}\textsuperscript{*} and Marina Rubini\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a} Department of Organic Chemistry, University of Konstanz, D-78464 Konstanz, Germany. E-mail: marina.rubini@uni-konstanz.de; Fax: +49 7531 884150; Tel: +49 7531 882398

\textsuperscript{b} Leibniz Institute of Molecular Pharmacology, D-13125 Berlin and Humboldt Universität zu Berlin, D-12489 Berlin, Germany. E-mail: hackenbe@fmp-berlin.de; Fax: +49 30 94793109; Tel: +49 30 94793181

\textsuperscript{c} Department of Biochemical Pharmacology, University of Konstanz, D-78464 Konstanz, Germany

*Corresponding authors:

Marina Rubini: marina.rubini@uni-konstanz.de

Christian Hackenberger: hackenbe@fmp-berlin.de

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1. Materials

CHO-EPO was purchased from Calichem; \(p\)-Azidophenylalanine was purchased by Bachem. The AlamarBlue® Reagent was from Life Technologies. Symmetrical PEG750-phosphites were synthesized according to published protocols.\(^1\)

**Plasmid and cell lines**

The plasmid pEVOL-pAzF was a gift from Peter Schultz (Addgene plasmid #31186).\(^2\) EPO gene sequences with an optimized *E.coli* codon usage were purchased from GeneArt. The sequence was cloned into pET11a (Novagen) using restriction sites BamHI/NdeI. The sequence of the EPO gene (without amber stop codons) is depicted below (restriction sites are underlined):

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1  ccatatggcac cgcctgtctgtatttggtagtcgagtcggttc tggacgtta tctgctggaa

61  gcacaaagaag cccgaaatat ctaccacggt tgtgcagaac attgtagcct gaatgaaa

121  attacagtgc cggataccaa agtgaatttt tatgctggga aacgtagtct cgtggtcag

181  caggcagtgg aagttttggca gggcttgga ctgcttgacg aagctgtct cgtggtcag

241  gcactgcttg ttaaaagcag ccagccgtgg gaaccgctgc agctgcatgt tgataaagca

301  gttagcggctc tgcgtagcct gaccaccctg ctgcgtgcac tgggtgccca gaaagaagca

361  atttctaaata gcgatgcagc atctgcagca ccgctgcgta ccattaccgc agataccttt

421  cgtaaacgtg ttcgcgtgta tagcaatttt ctgcgtggca aactgaaact gtataccggt

481  gaagcatgtc gtacggtgtga tgcgtcac catcapcatc attaaggac c
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The codons for Lys (AAA) in the box were singly mutated to the amber stop codon UAG.

The *E. coli* strain BL21(DE3) was used for the expression of all EPO variants.
2. Supplementary Figures

Supplementary Figure 1

Supplementary Figure 1. 15% SDS-PAGE. Expression profile of EPOpAzF in whole cell lysate. n.i.: not induced sample.
**Supplementary Figure 2.** 15% SDS PAGE showing the conversion of Staudinger-phosphite reaction between EPOpAzF and PEG750-phosphite (top) and EPO24pAzF and PEG2000-phosphite (bottom).
Supplementary Figure 3. 15% SDS-PAGE showing the isolation of EPO24pAzF-PEG750 (top) and EPO24pAzF-PEG2000 (bottom) from the unPEGylated form after Gel filtration.
Supplementary Figure 4. MALDI-TOF Spectrum for PEGylated EPO24pAzF-PEG750
Supplementary Figure 5. Melting curves of EPO variants containing pAzF at different positions and their PEGylated analogs. Melting curves were recorded by following the decrease in the ellipticity at 220 nm during temperature increase. EPO83pAzF could not be isolated in sufficient amounts for characterization, due to extreme aggregation propensity.
Supplementary Figure 6. Far-UV Circular Dichroism (CD) spectra of PEGylated EPO variants (original data)
Supplementary Figure 7. *In vitro* bioactivity of EPO24pAzF, EPO38pAzF, and EPO83pAzF measured by cell proliferation assay with TF-1 cells. The relative increase in cell number was plotted against EPO concentration, and the data were fitted to a non-cooperative binding reaction with a single binding site (Hill coefficient = 1) (solid lines).
Supplementary Figure 8

Supplementary Figure 8. Percent of BFU-E colonies after treatment with 50ng/mL EPO variants on mouse bone marrow cells.
References
