UNIVERSITY OF BUCHAREST
DEPARTMENT OF CHEMISTRY

Ph.D THESIS

ABSTRACT

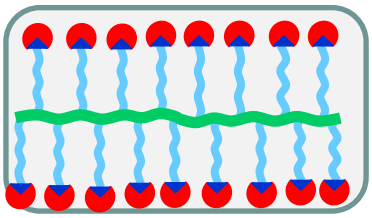
Synthesis of new inhibitors for enzymes and cellular defective enzyme systems. Applications in biochemistry and macromolecular chemistry.

Scientific advisor,
Prof. Dr. Ion Baciu

Ph.D student,
Dumitru Ioana
(cas. Nicolau)

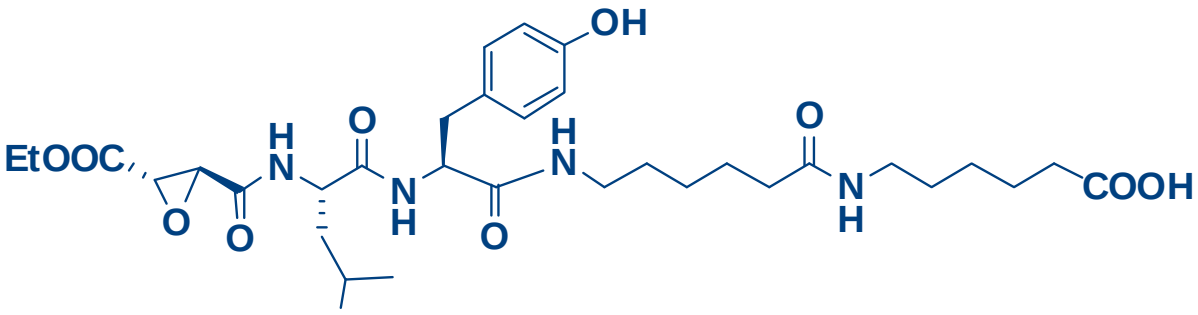
2012

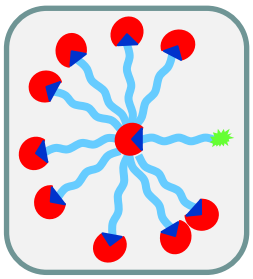
Table of Contents



PART A.

Peptidyl-epoxysuccinates – ABPs with direct applications in the macromolecular chemistry





PART A.

In this part we planed to make protein-based polymers and dendrimers taking advantage of the unique property of activity-based probes to label active enzymes specifically at an amino-acid position in the active site of the enzyme (Figure A.24 and A. 26).

When the activity-based probe is decorated with a polymerisable group (Figure A.29 a) one obtains a "polymerisable inactivated enzyme" (Figure A.24).

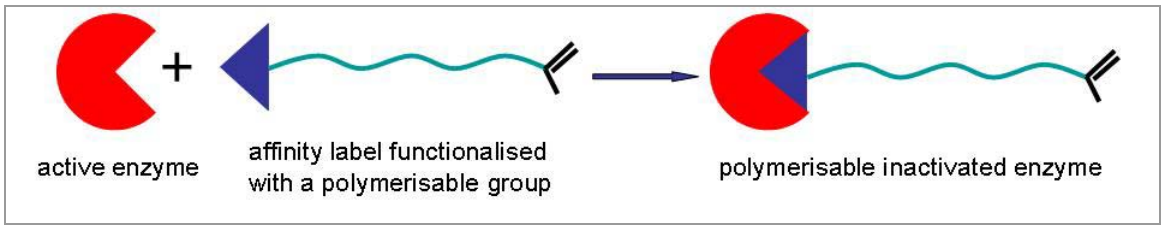


Figure A. 24: *The specific reaction of the activity-based probe with an active enzyme yields an inactivated enzyme that is functionalised at a specific amino-acid with a polymerisable group*

After the (co)polymerisation process one would obtain a polymeric chain on which "hang" protein molecules, all of which are attached through the same amino-acid (Figure A.25).

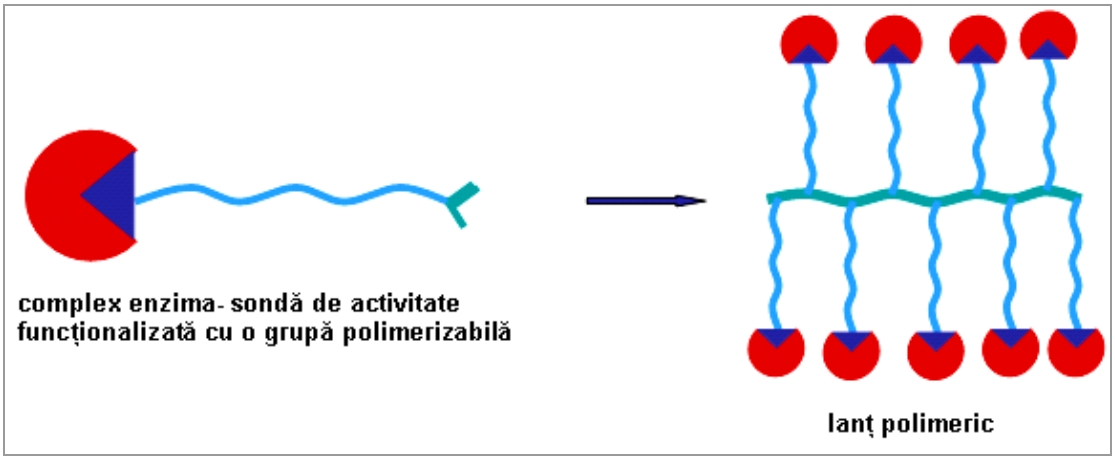


Figure A. 25: *When the "polymerisable inactivated enzyme is submitted to the (co)polymerisation reaction, one would obtain a protein-functionalised polymer*

When the activity-based probe is decorated with a NHS residue (Figure A.29 c) one obtains a inhibited enzyme "decorated" on her surface with ABPs (Figure A.26) and after another reaction with an activated enzyme one obtains the new proteic dendrimer (Figure A.26).

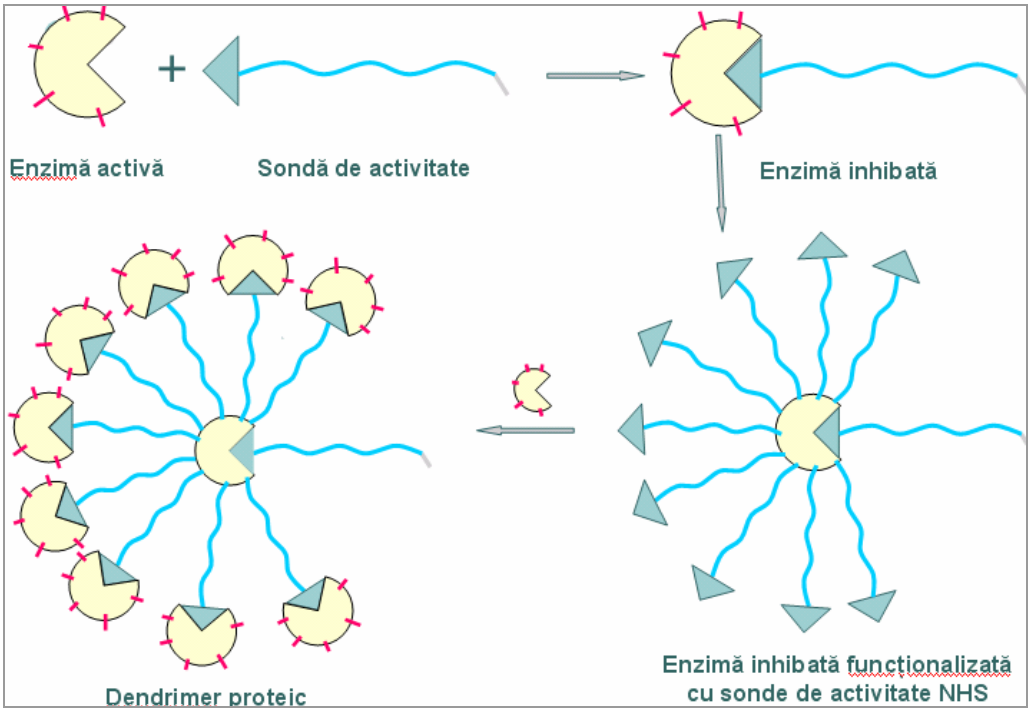


Figure A.26. *Synthesis pathway of proteic dendrimers*

The activity-based probes can be generally defined as reagents that meet the following criteria:

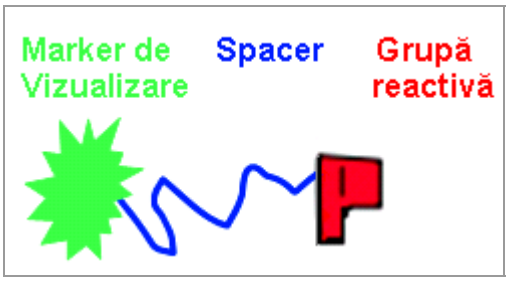
1. React with a broad range of enzymes from a particular class directly in complex proteome.
2. React with these enzymes in a manner that correlates with their catalytic activities.
3. Display minimal cross-reactivity with other protein classes.
4. Posses a tag for the rapid detection and in some cases isolation of the reactive enzymes.

An **activity-based probe** consists of three distinct functional elements (Figure A.11): a **reactive group** for covalent attachment to the enzyme of interest, a **linker region** that can modulate reactivity by providing spacing and specificity of the reactive group and a **tag** for identification and/or purification of the modified enzymes.

The reactive group must contain a functional group that is *reactive* toward the specific residue on the target protein and *inert* toward other reactive species within the cell or cell extracts. It can take the form of an epoxide, fluromethyl ketone, acyloxymethyl ketone, vinyl sulfone etc. for covalent binding of cysteine prooteases (Figure A.11.B. c). The linker region connects the reactive group to the tag used for identification/purification (Figure A.11.B. b) and can take the form of a long-alkyl chain, polyethyleneglycol spacer, a peptide or peptide-like structure. The tags are usually affinity, fluorescent or radioactive molecules which allow a quick

visualization of the probe-modified protein or purification from a complex matrix (Figure A.11.B. a).

A)



B)

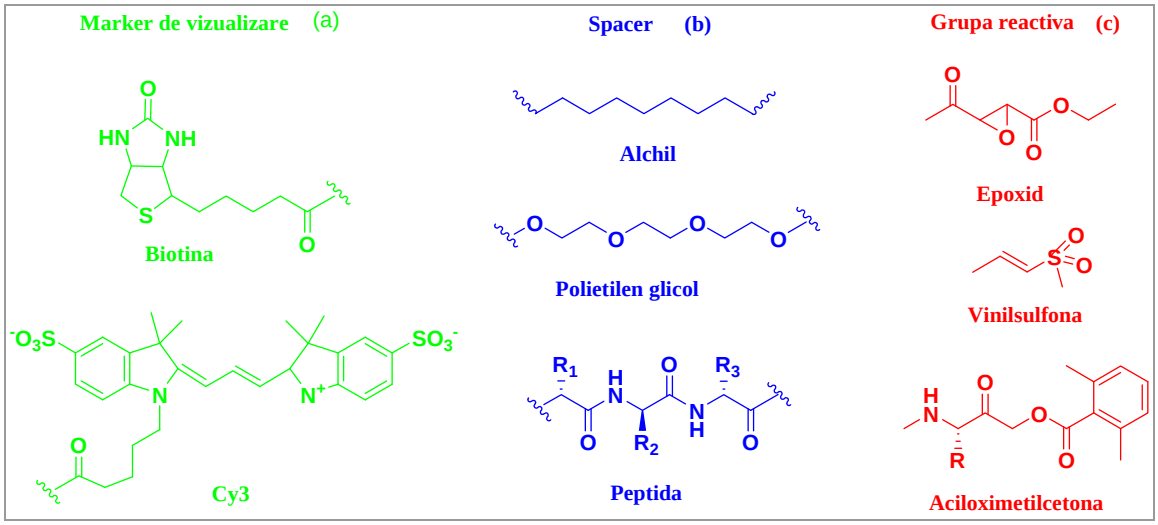
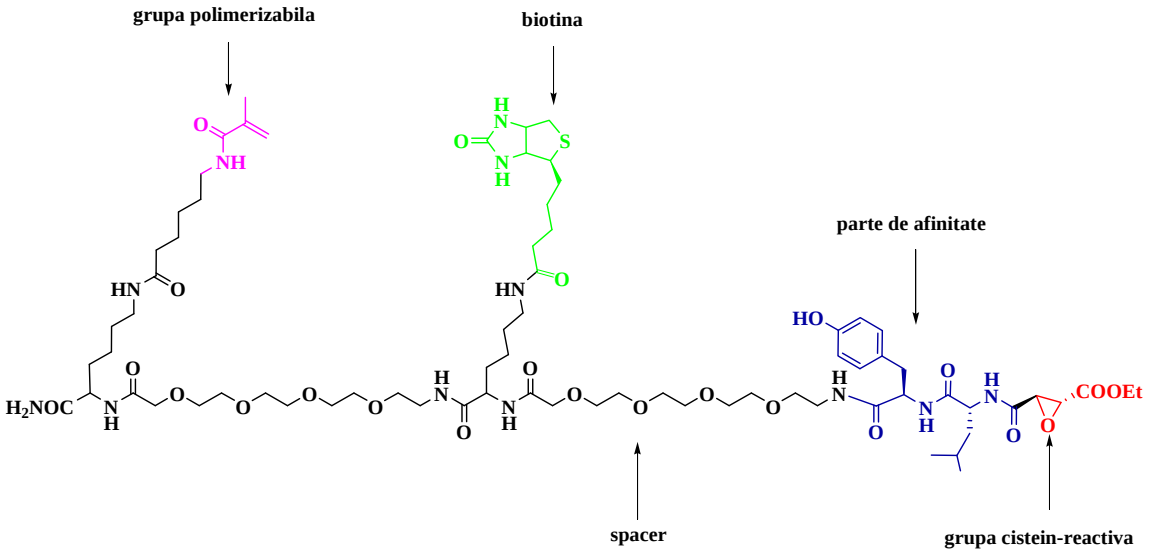
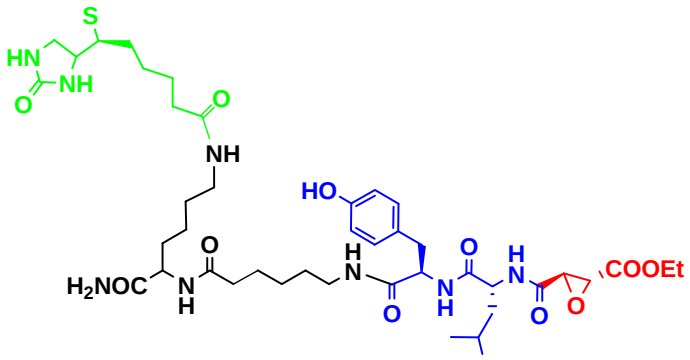


Figure A.11. *The structure of ABPs: A) schematic representation, B) Constitutive elements*

We synthesized a series of ABPs that mimics the structures presented in the figure A.29 a)



b)



c)

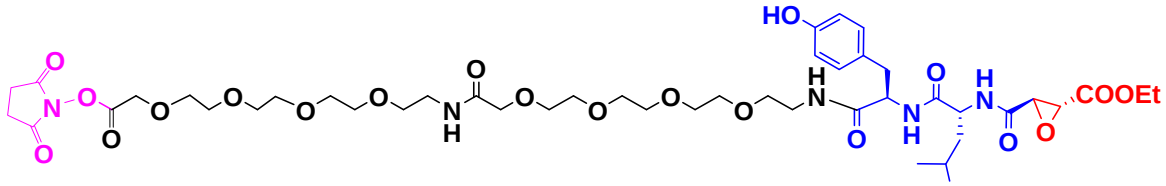


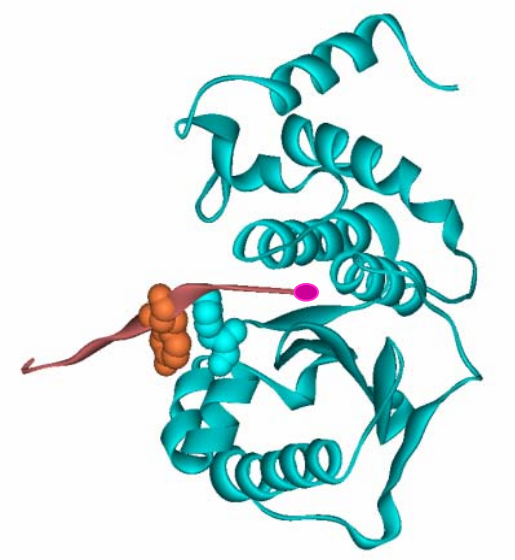
Figure A.29. *ABP decorated with a) polimerysable group; b)visualization marker; c) NHS residue.*

As protein we used papain. The active site of papain contains a catalytic triad of ^{25}Cys, ^{159}His and ^{175}Asn. ^{175}Asn has been proposed to orientate ^{159}His, so that the imidazole group of ^{159}His polarizes the thiol group of ^{25}Cys, thus allowing deprotonation even at neutral to weakly acidic pH. Therefore, the epoxide-based mechanism makes use of an electrophilic carbon that is susceptible to attack by the active-site nucleophile of the enzyme. It results the formation of a covalent bond between the electrophilic carbon in the epoxide ring and the active-site thiol residue.

Using an innovative concept, thus principal was exposed in chapter 2, we synthesized new proteic based polymers and dendrimers. The compounds where analyzed through SDS-PAGE and MALDI-MS.

PART B.

Glycin-fluoromethyl ketone – ABP for sentrin proteases



PART B.

SUMO (Small Ubiquitin-like MOdifier) is an ubiquitin-like protein that regulates the function and localization of a growing number of cellular proteins. SUMOylation is reversed by a class of cysteine protease called SENPs (Sentrin/SUMO specific proteases). SENPs are involved in the cleavage of SUMO variants from SUMO-conjugated proteins (isopeptidase activity) and in the maturation of SUMOs (endopeptidase activity)[1] (figure B.6)

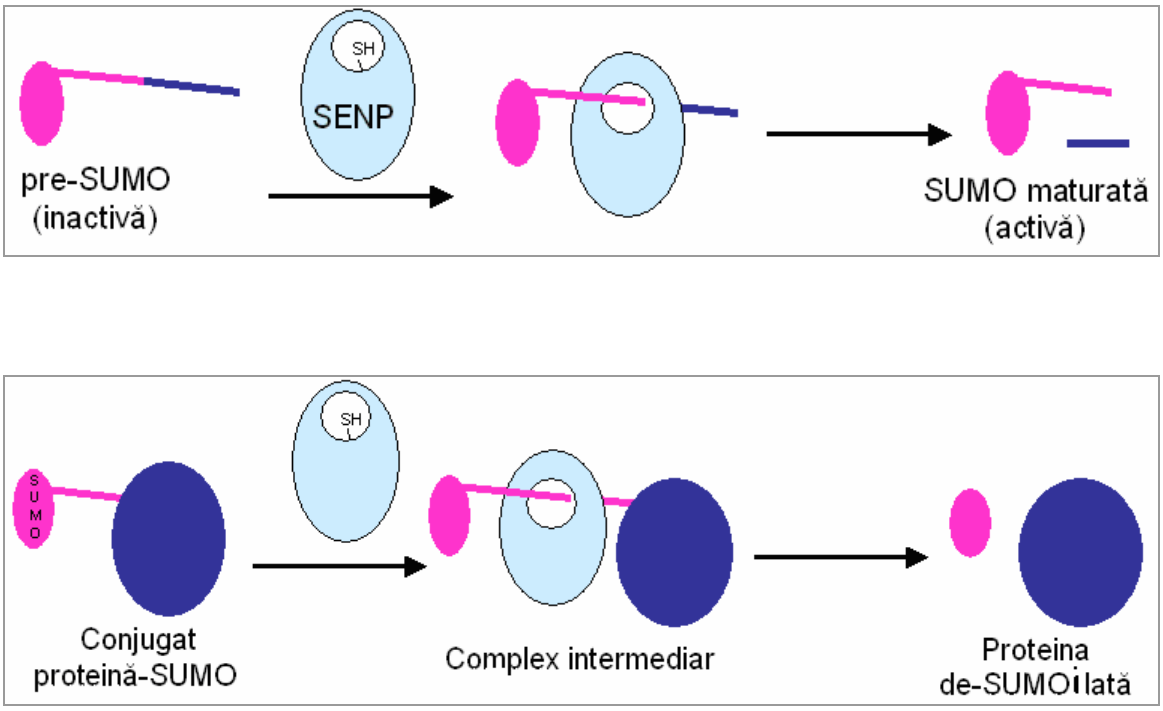


Figura B.6. *SENPs functions.*

Recent studies have sought to define the substrate specificity that distinguishes individual SENPs[2]. To gain further insight into the functional role of SENPs as well as to facilitate the discovery of natural and artificial inhibitors for SENPs we sought to design small-molecule activity-based probes that have proven very informative for other protease families

In the design of activity-based probes for SENPs, there are two *a priori* conditions to be followed. Firstly, crystallographic studies reveal that, SENPs possess a tunnel-like cavity in their active sites[3], by positioning three residues (Trp, Val and active site cysteine) on two proximal loops of the protein

[1] S. J. Li, M. Hochstrasser, *Nature*, **1999**, *398,* 246-251; b) L. Gong, E. T. Yeh, *J. Biol. Chem.,* **2006**, *281*, 15869-15877; c) M. Drag, G.S. Salvesen, *Life*, **2008**, *60*, 734-742; d) E. T. H. Yeh, *J. Biol. Chem.* **2009**, *284*, 8223–8227; e) D. Mukhopadhyay, M. Dasso, *Trends Biochem. Sci.*, **2007**, *32*, 286-285; Kim, J. H., Baek, S. H., *Biochim. Biophys. Acta,* **2009**, *1792*, 155-162.

[2] a) M. Drag, J. Mikolajczyk, I. M. Krishnakumar, Z. Huang, G. S. Salvesen, *Biochem. J.,* **2008**, *409*, 461-469; b) J. Mikolajczyk, M. Drag, M. Bekes, J. T. Cao, Z. Ronai, G. S. Salvesen, *J. Biol. Chem*., **2007**, *282*, 26217-26224; c) L. N. Shen, M. -C. Geoffroy, E. G. Jaffray, R. T. Hay, *Biochem. J.* **2009**, *421*, 223-230.

[3] a) Z. Xu, S. F. Chau, K. H. Lam, H. Y. Chan, T. B. Ng, S. W. N. Au, *Biochem. J.*, **2006**, *398*, 345-352; b) L. N. Shen, C. Dong, H. Liu, J. H. Naismith, R. T. Hay, *Biochem. J.*, **2006**, *397*, 279-288.

structure (Figure B.15).

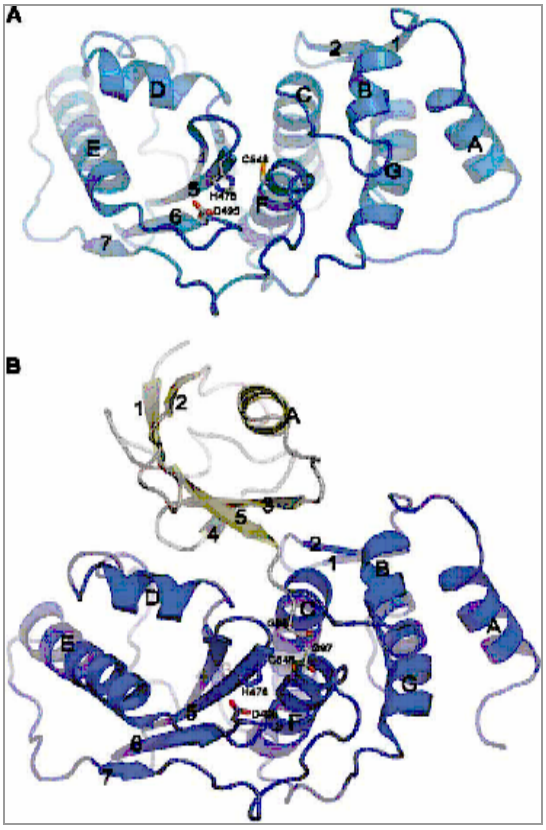


Figure B.15. *SENP2 C-terminal domain (**A**) and SENP2-SUMO1 C-terminal domain (**B**)(www.pdb.org)*

This drives accommodation of only a *C*-terminus Gly-Gly sequence in the substrate cleft, making access of the most electrophile, cysteine-reactive groups to the catalytic site difficult. Secondly, one has to find a short peptide sequence with sufficient binding strength for SENPs, so that the probe is reactive with SENP proteins at reasonable concentrations.

Thus we decided to use a glycine fluoromethyl ketone (G-FMK) as a *C*-terminal functionality, since the SUMOs C-terminal sequence is composed of two glycines and since FMKs are quite close to parent amino acids in terms of bulkiness. Also SUMOs contain a conserved aromatic residue at the 7th position that appears to mediate a stacking interaction with a conserved aromatic residue of the corresponding SENP[4]. The mutation of this aromatic residue in Ulp1 (that cleaves Smt3p conjugates) is lethal in yeast[5].

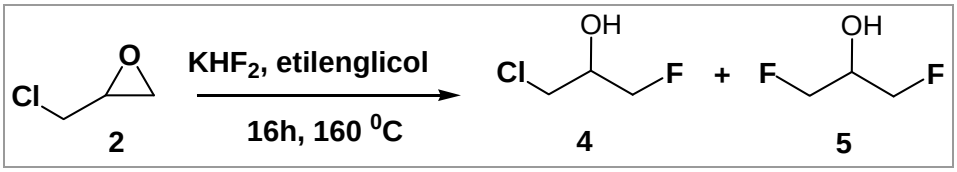
Based on these two hypotheses, we synthesized compound **1** and tested its binding to SENPs. Compound **1** contains as electrophile a glycine fluoromethyl ketone and, as a SENP specificity moiety, the peptide sequence FQQQTGG of SUMO2 (identical to the one in SUMO3), a natural substrate for both SENP1 and SENP2.

---

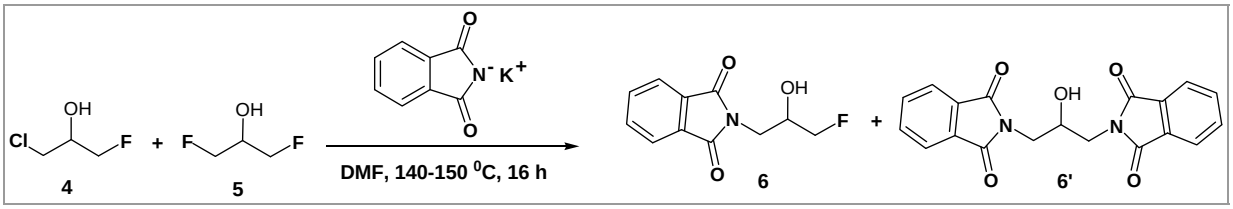
[4] L. N. Shen, C. Dong, H. Liu, J. H. Naismith, R. T. Hay, *Biochem.J.,* **2006**, *397*, 279-288
[5] E. Mossessova; C. D. Lima, *Mol. Cell,* **2000**, *5*, 865-876

The synthetic pathway to prepare target compound is described below (Scheme B.6, B.7, B.8 and B.10).

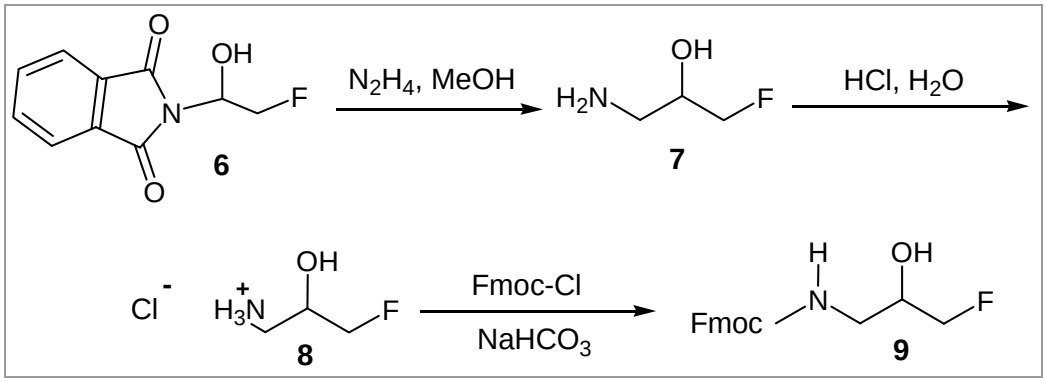
Epichlorhydrin **2** is ring-opened with potassium difluoride (KHF_2) and the resulting chloro-fluoro-alcohol **4** is converted to *N*-phthalimid-fluoro-alcohol **6** using potassium phthalimide (PhtK). Phthalimide removal with hydrazine hydrate yields fluoro-amino alcohol **7** which is further Fmoc protected to provide compound **9** in a reasonable yield. Dess-Martin oxidation of **9** yields fluoromethyl ketone **11**.



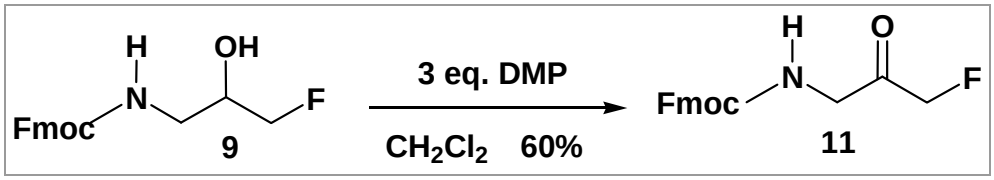
Scheme B.6. *Epichlorhydrin nucleophilic substitution*



Scheme B.7.*Converion of chloro-fluoro-alcohol to N-phthalimid-fluoro-alcohol*

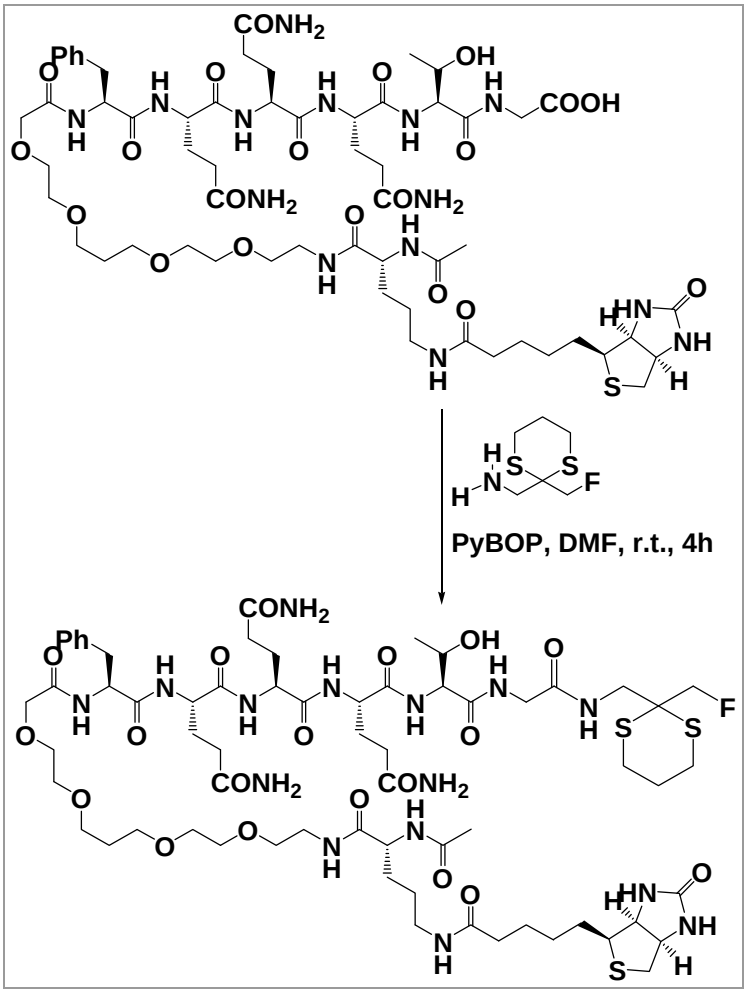


Scheme B.8. *Fmoc-amino-fluoro-alcohol synthesis*



Scheme B.10. *Fmoc-fluoro-alcohol oxidation*

Fluoromethyl ketone **11** is further treated with silylated 1,3-propanedithiol and Fmoc deprotected to yield dithiane amine. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluoro-phosphate (PyBOP) mediated solution-coupling of dithiane amine with the carboxy-terminated peptide Biotin-Teg-FQQQTG-COOH (Teg=tetraethyleneglycol spacer[6]) gives the protected fluoromethyl peptide (Scheme B.20).

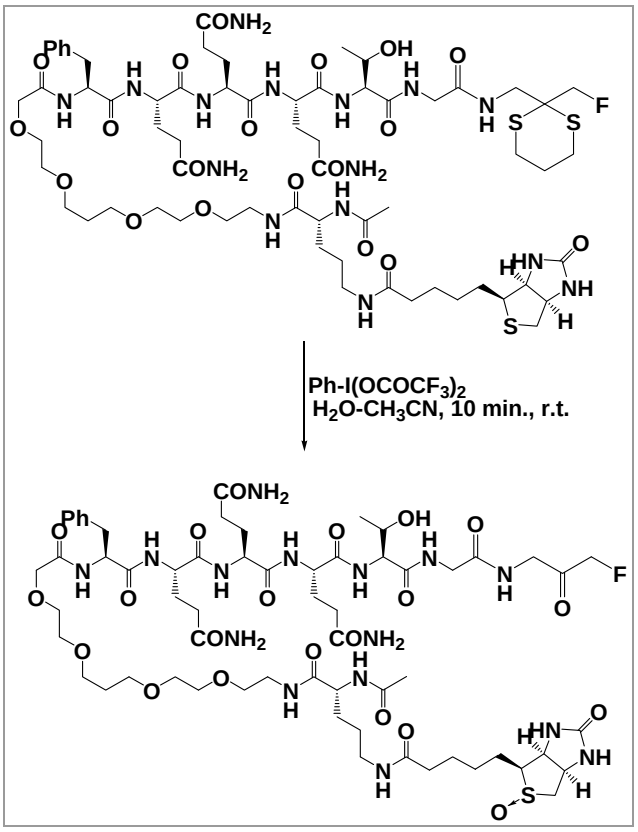


Scheme B.20. *Coupling of dithiane amine with the carboxy-terminated peptide Biotin-Teg-FQQQTG-COOH*

In the final step dithiane is deprotected with bis(trifluoroacetoxy)iodobenzene (BTI) to provide peptidyl fluoromethyl ketone **1.** (Scheme B.21)

It is to be noted that BTI oxidizes also the terminal biotin, to a mixture of α and β sulfoxides, fact that is inconsequential with the biotin-detection methods employed, since the biotin sulphoxides are known to bind streptavidin with sufficient affinity for detection purposes[7].

[6] N. D. Bogdan, M. Matache, V. M. Meier, C. Dobrotă, I. Dumitru, G. D. Roiban, D. P. Funeriu, *Chem. Eur. J.* **2010**, *16*, 2170-2180.
[7] a) R. K. Garlick, R. W. Giese, *Biochem. J.*, **1990**, *268*, 611-613; b) H. C. Lichstein, J. Birnbaum, *Biochem. Bioph. Res. Co.*, **1965**, *20*, 41-45.



Scheme 2.21. *Dithian deprotection*

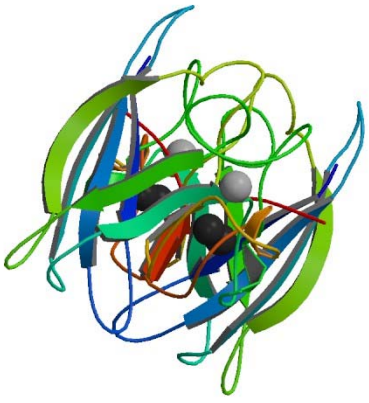
Stability studies conducted on *N*-acetylglycine fluoromethylketone, by monitoring its ^{19}F NMR signal, showed that the compound is stable to hydrolysis for more than one hour at room temperature in a buffer that is relevant for SENP labelling reactions (25 mM Tris/HCl, pH 7.5, 0.8 M sodium citrate).

Conclusions

We describe and characterise the properties of a new class of compounds that can act as activity-based probes for SENP1 and SENP2. Before compound **1**, the only known probes specific for SENPs were obtained adding to the C terminus of SUMO moieties an electrophilic trap[14]. To our knowledge compound **1** is the first small probe targeting SENPs.

PART C.

Cu (II) complexes – superoxid dismutase 1 (SOD1) mimetics



PART C.

Superoxide dismutases (SODs) stand in the prime line of enzymatic antioxidant defense in nearly all eukaryotic cells exposed to oxygen, catalyzing the break-down of the superoxide anionic radical to O_2 and H_2O_2.

Overproduction of superoxide correlates with numerous pathophysiological conditions, and although the native enzyme can be used as a therapeutic agent in superoxide-associated conditions, synthetic low molecular weight mimetics are preferred in terms of cost, administration mode, and bioavailability. In this study we make use of the model eukaryote Saccharomyces cerevisiae to investigate the SOD-mimetic action of a mononuclear mixed-ligand copper(II) complex, [CuCl(acac)(tmed)] (where acac is acetylacetonate anion and tmed is N,N,N',N'-tetramethylethylenediamine).

Taking advantage of an easily reproducible phenotype of yeast cells which lack Cu–Zn SOD (Sod1p), we found that the compound could act either as a superoxide scavenger in the absence of native Sod1p or as a Sod1p modulator whice behaved differently under various genetic backgrounds.

Oxygen is essential for aerobic life, but it can also have deleterious effects owing to generation of reactive oxygen species (ROS) during aerobic metabolism and mitochon-
drial respiration[8]. Excessive ROS formation can lead to oxidative stress, causing cell injury due to oxidative attack on DNA, proteins, unsaturated lipids, and other biomolecules[9]. Superoxide dismutase is a key antioxidant enzyme present in all aerobic organisms, and catalyzes the dismutation of superoxide to O2 and H2O2. The superoxide anionic radical is a biologically important ROS which is formed from various sources, including normal mitochondrial respiration and the activity of several enzymes, such as xanthine oxidase[10]. In humans, superoxide overproduction is proposed to contribute to inflammation, modified cellular metabolism, and tissue injury in diabetes, stroke, cardiovascular disease, and cancer as well as in injuries related to radiation[11]. To defend themselves against the deleterious effects of superoxide, most eukaryotic cells possess two evolutionarily distinct forms of SOD: a copper- and zinc-containing SOD (Cu,Zn-SOD; SOD1) found predominantly in the cytosol[12] and a manganese-containing SOD (Mn-SOD; SOD2) that localizes strictly to the mitochondrial matrix[13]. Protective and beneficial roles of SOD have been demonstrated in a broad range of diseases, both preclinically and clinically[14]. Although attempts to use SOD as a therapeutic agent have been made, the therapeutic effects of

---

[8] a) Longo VD, Gralla EB, Valentine JS, *J Biol Chem,*, **1996**, 271, 12275, b) Valentine JS, Wertz DL, Lyons TJ, Liou LL, Goto JJ, Gralla EB *Curr Opin Chem Biol*, **1998**, 2, 253

[9] Fridovich I, *Science*, **1978**, 201, 875, b) Emerit J, Michelson AM, *Sem Hop*, **1982**, 58, 2670, c) Stadtman ER, *Annu Rev Biochem*, **1993**, 62, 797

[10] Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H, *Free Radic Biol Med*, **2007**, 43, 477

[11] a) Finkel T, *Curr Opin Cell Biol*, **2003**, 15, 247 b) Liu H, Colavitti R, Rovira II, Finkel T, *Circ Res,* **2005**, 97, 967 c) McCord JM, Edeas MA, *Biomed Pharmacother*, **2005**, 59, 139

[12] McCord JM, Fridovich I, *J Biol Chem*, **1969**, 244, 6049

[13] Weisiger RA, Fridovich I, *J. Biol Chem*, **1973**, 248, 4793

[14] a) Halliwell B, Gutteridge JMC, **1985**, In: Baum H, Gergely J, Fanburg BL (eds) Free radicals in biology and medicine. Oxford University Press, Oxford, pp 89–193 b) Mc Cord JM, *Science*, **1974**, 185, 529 c) Maxwell SR, *Drugs*, **1995**, 49, 345

exogenous native SOD are modest[15]; therefore, interest in synthetic compounds which modulate the SOD response or even mimic the SOD activity has been increasing constantly[16]. In particular, Cu,Zn-SOD has been proposed for clinical uses[17], but this enzyme has several pharmacological limitations, such as high cost, low lipid solubility, low penetration into cells, immunogenicity, and lability from the action of gastric and intestinal proteases[18]. To overcome these problems, low molecular weight metallic complexes can be used as SOD mimetics with potential as pharmacological agents and radioprotectants[19]. Although many Cu(II) or Mn(III) complexes with SOD-mimetic action have been reported[20], in vivo studies demonstrating their activity are still scarce. Moreover, most of the SOD mimics are not well tolerated by living organisms, being more or less toxic[21].

In this report, we describe the use of the model eukaryotic microorganism Saccharomyces cerevisiae for testing the Sod1p-mimetic or Sod1p-modulator activity of a Cu(II) mixed-ligand complex, and also attempt to understand the conditions under which a potential helpful compound becomes a villain and toxic to cells. The yeast has proved to be a very useful eukaryotic model for studies of the effects of small molecules at the cellular level and an attractive alternative to mammalian cell lines, and especially to the controversial experiments on animals. Such studies not only verify the in vitro results of test-tube experiments on the protective effect, but also reveal the possible side effects and the products of their metabolism.

Yeast cells are thought to generate ROS through the same mechanisms as mammalian cells and express many of the same antioxidant factors[22]. Like most eukaryotes, the baker's yeast S. cerevisiae contains two intracellular superoxide dismutases: Cu,Zn-SOD (Sod1p, encoded by gene SOD1), which is predominantly localized in the cytosol[23], and Mn-SOD (Sod2p, encoded by gene SOD2), which is localized to the matrix of the mitochondria[24]. Deletion of SOD1 correlates to a number of oxygen-dependent growth defects of yeast cells, including oxygen sensitivity, slow growth, hypersensitivity to superoxide-generating agents (e.g., paraquat, menadione), accelerated aging, and the quite-at-hand auxotrophy for methionine and lysine[25]. These amino acid biosynthetic defects do not occur under

---

[15] a) Afonso V, Champy R, Mitrovic D, Collin P, Lomri A, *Joint Bone Spine*, **2007**, 74, 324 b) Levin ED *Curr Alzheimer Res*, **2005**, 2, 191

[16] a) Pong K, *Expert Opin Biol Ther*, **2003**, 3, 127 b) Muscoli C, Cuzzocrea S, Riley DP, Zweier JL, Thiemermann C, Wnag ZQ, Salvemini D, *Br J Pharmacol*, **2003**, 140, 445

[17] Emerit J, Pelletier S, Likforman J, Pasquier C, Thuillier A, *Free Radic Res Commun*, **1991**, 12–13, 563

[18] Rao VS, Goldstein S, Czapski G, *Free Radic Res Commun*, **1991**, 12–13, 67

[19] Batinic ´-Haberle I, Rebouc ¸as JS, Spasojevic ´ I, *Antioxid Redox Signal*, **2010**, 13, 877

[20] Saczewski F, Dziemidowicz-Borys E, Bednarski PJ, Gdaniec M, *Arch Pharm,* **2007**, 340, 333 b) Barik A, Mishra B, Kunwar A, Kadam RM, Shen L, Dutta S, Padhye S, Satpati AK, Zhang HY, Priyadarsini KI, *Eur J Med Chem*, **2007**, 42, 431

[21] Potapov AS, Nudnova EA, Domina GA, Kirpotina LN, Quinn MT, Khlebnikov AI, Schepetkin IA, *Dalton Trans*, **2009**, 23, 4488

[22] a) Jamieson DJ, *Yeast*, **1998**, 14, 1511 b) Carmel-Harel O, Storz G, *Annu Rev Microbiol,* **2000***,* 54, 439

[23] Crapo JD, Oury T, Rabouille C, Slot JW, Chang LY , *Proc Natl Acad Sci USA*, **1992**, 89, 10405

[24] Weisiger RA, Fridovich I, *J Biol Chem*, **1973**, 248, 4793

[25] Gralla EB, Kosman DJ, *Adv Genet*, **1992**, 30, 251 b) Culotta VC, *Curr Top Cell Regul*, **2000**, 36, 117 c) Sturtz LA, Culotta VC, *Methods Enzymol*, **2002**, 349, 167

anaerobic conditions and appear to be the result of endogenously generated ROS[26], representing a convenient model to investigate putative Sod1p mimics or Sod1p modulators.

Extensive work has been done on S. cerevisiae SODs[27] as well as on the possibilities to use yeast SOD mutants to reveal the mechanism of SOD-related diseases[28] or to test the therapeutic activity of various molecules[29]. In this work we took advantage of the lysine auxotrophy of a yeast sod1D knockout mutant to investigate the Sod1p-like activity of a Cu(II) coordination compound with mixed ligands, [CuCl(acac)(tmed)] (Fig. 1B). This compound belongs to the class of mono-nuclear mixed-ligand Cu(II) complexes of the general formula [CuX(OO)(NN)] (where OO stands for a monoanionic bidentate oxygen-donor ligand and NN represents a chelating diamine ligand), in which there is great interest owing to their significant pharmacological properties[30], and studies regarding correlation between their molecular structure and biological activity have been done[31]. A synthetic procedure to obtain the [CuCl(acac)(tmed)] complex has been previously reported along with its crystal structure[32].

The [CuCl(acac)(tmed)] complex exhibits in vitro SOD-like activity

Complexes of the general formula [CuX(OO)(NN)] possessing a square-pyramidal environment for the Cu(II) ion with the basal plane defined by the donor atoms of the chelating ligands (Fig. C.12A) have potential SOD-like activity based on a Cu^{+}/Cu^{2+} redox cycling. A member of this group, [CuCl(acac)(tmed)] (Fig. 1B), was assayed in vitro for SOD-like activity. A simple and rapid qualitative test was developed by placing a solution of the compound tested onto a filter disk pretreated with NBT.

[26] Liu XF, Elashvili I, Gralla EB, Valentine JS, Lapinskas P, Culotta VC, *J Biol Chem*, **1992**, 267, 18298
[27] Leitch JM, Yick PJ, Culotta VC, *J Biol Chem*, **2009**, 284, 24679
[28] Bastow EL, Gourlay CW, Tuite MF, *Biochem Soc Trans*, **2011**, 39, 1482
[29] Zyracka E, Zadrag R, Kozioł S, Krzepiłko A, Bartosz G, Bilin ´ski T, *Acta Biochim Pol*, **2005**, 52, 679
[30] a) De Vizcaya-Ruiz A, Rivero-Muller A, Ruiz-Ramirez L, Kass GEN, Kelland LR, Orr RM, Dobrota M, *Toxicol Vitr*, **2000**, 14, 1 b) Garcia-Mora I, Ruiz-Ramı ´rez L, Go ´mez-Ruiz C, Tinoco-Me ´ndez M, Marquez-Quinones A, *Metal Based Drugs*, **2001**, 8, 19
[31] Bravo-Go ´mez ME, Garcı ´a-Ramos JC, Garcia-Mora I, Ruiz-Azuara L, *J Inorg Biochem* , **2009**, 103, 299 b) Onawumi OOE, Odunola OA, Suresh E, Paul P, *Inorg Chem Commun*, **2011**, 14, 1626
[32] Jalilehvand F, Ishii Y, Hidai M, Fukuda Y , *J Chem Soc Dalton Trans*, **1996**, 3251

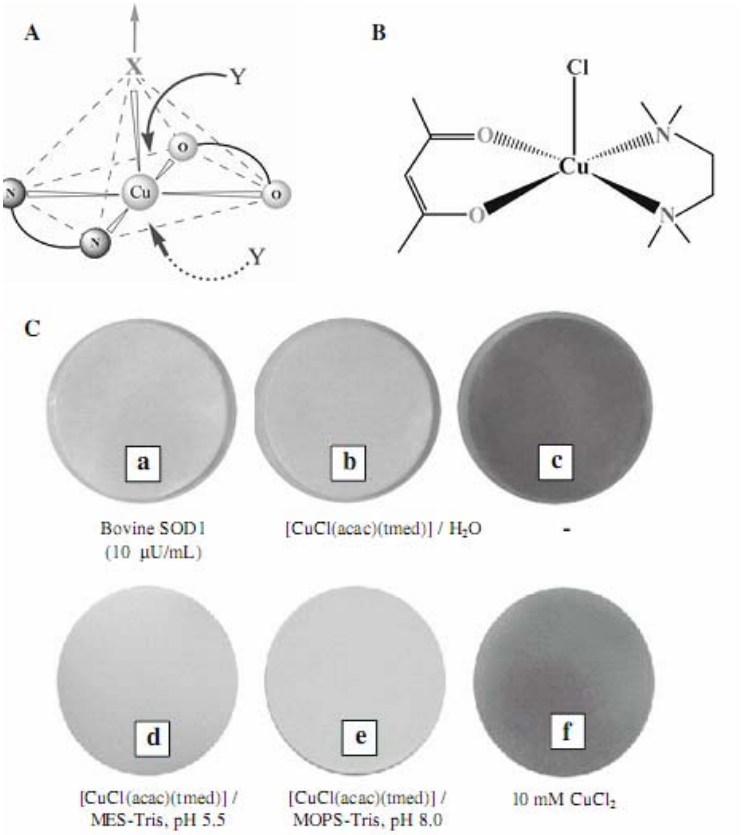


Figure C.12. Structure of the mononuclear Cu(II) complex used in this study. A The square-pyramidal coordination geometry of the compound allows the substitution of the X. B The structure of [CuCl(acac)(tmed)]. C [CuCl(acac)(tmed)] exhibits SOD-like activity in vitro similarly to bovine Cu,Zn-SOD.

When superoxide ions are produced through exposure to light, NBT is reduced to the formazan dye and the disk turns from white to purple-blue (Fig. C.12C, c). Aqueous solutions of [CuCl(acac)(tmed)] inhibited the reduction of NBT (Fig. C.12.C, b) similarly to a solution of commercial bovine Cu,Zn-SOD (Fig. C.12.1C, a), presumably by scavenging the O21- anionic radical. The test was repeated under various pH conditions, and similar activity was noted when the compound was dissolved in 10 mM MES/Tris (pH 5.5–6.7) or MOPS/Tris (pH 6.7–8). Tests were performed in buffers of increasing pH (0.1 increments) with similar results; only data for the extreme pH values used are shown (Fig. C.12C, d, e). In contrast, free $Cu2^{+}$ ions showed a muchlower discoloring activity (Fig. C.12.C, f), suggesting that the superoxide-scavenging activity of the compound was due to the complex itself, and not the result of dissociation in the aqueous environments. Also, solutions of the free ligands (tmed and acac) showed no SOD-like activity in the filter disk assay.

CuCl(acac)(tmed)] alleviates the lysine auxotrophy of a sod1D yeast mutant

As [CuCl(acac)(tmed)] inhibited the reduction of NBT in vitro, the question was raised whether this compound could act as a SOD mimic within the living cell. To test this possibility, we took advantage of the lysine auxotrophy exhibited by the S. cerevisiae cells knocked out for SOD1, the gene known to

encode Cu,Zn-SOD in yeast (Sod1p).

When they were grown on lysine-free medium supplemented with [CuCl(acac)(tmed)], the sod1D cells could overcome the growth defect caused by the absence of lysine (Fig. C.14a). Supplementing the medium with equimolar concentrations of tmed, acetylacetone, or CuCl2 had no effect on the lysine auxotrophy of the sod1D mutant, indicating that the rescuing ability was due to the complex itself and not due to the components resulting from dissociation of the complex. Remarkably, the sod1D cells could grow better than the wild-type or the sod2D cells on SD-K plates supplemented with the compound (Fig. C.14a). This phenotype was also noticed when the sod1D cells were grown in liquid SD-K supplemented with [CuCl(acac)(tmed)] in concentrations between 0.05 and 0.5 mM (Fig. C.14b), suggesting that the compound may be used by the cell as a protectant against the ROS (namely, O21-) that lead to defects in the lysine biosynthetic pathway.

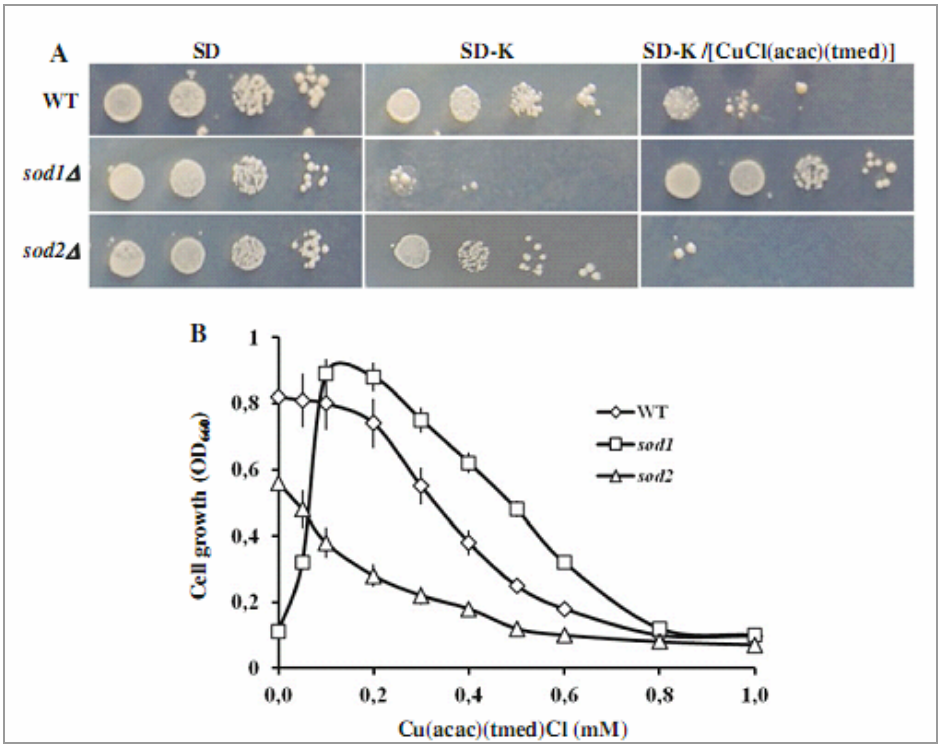


Fig. C.14 [CuCl(acac)(tmed)] alleviates superoxide-related growth defects of yeast cells lacking SOD1. a The lysine auxotrophy of sod1D cells is abolished by [CuCl(acac)(tmed)]. b [CuCl(acac)(tmed)] not only alleviates the lysine auxotrophy, but also improves the growth of the sod1D cells.

[CuCl(acac)(tmed)] has SOD-like activity, but also modulates the Sod1p activity within yeast cells

As [CuCl(acac)(tmed)] alleviated the lysine auxotrophy of sod1D cells, this suggested that the compound could act as a scavenger for superoxide in the absence of Sod1p. To check this possibility, the capacity of the compound to alleviate the toxicity of superoxide-generating agents was tested. For this purpose, mid-log-phase cells were spread on agar plates containing toxic, but sublethal concentrations of paraquat (PQ; 0.1 mM), and 3 lL of [CuCl(acac)(tmed)] aqueous solution (1 mM) was spotted onto the center of the plate.

After 3 days' incubation under normal aeration, it was noted that the compound had the ability to diminish the toxicity of PQ toward the sod1D cells (Fig. C.15b, middle). Surprisingly, under the same conditions, the compound had no visible effect on the growth of wild-type cells (Fig. C.15b, left), but it impaired the growth of sod2D cells (Fig. C.15b, right). Similar results were obtained when the media were supplemented with another superoxide-generating agent, menadione (data not shown). The three cell lines also exhibited divergent tolerance toward the compound even in standard YPD medium. Thus, the compound seemed to affect the growth of both wild-type and sod2D (with null mutation in the gene encoding the mitochondrial Sod2p) cells, while having beneficial effects on the growth of sod1D cells (Fig. C.15a). When equimolar (or lower) concen- trations of Cu^{2+} or tmed were used instead of [CuCl(acac)(tmed)], no effect was detected, indicating that the phenotypes described above were not the result of the dissociation of the coordination compound.

As [CuCl(acac)(tmed)] alleviated the lysine auxotrophy of the sod1D mutant, the influence of the compound on Sod1p activity was determined in the cytosolic extracts obtained from the yeast cells. Wild-type, sod1D, and sod2D cells were grown in the presence of nontoxic concentrations of the compound, and the Sod1p activity was monitored directly in gel, following nondenaturating polyacrylamide gel electrophoresis of the cell extracts. In the wild-type cells, the Sod1p activity was augmented by the presence of the compound in the growth medium (Fig. C.15c). The compound also augmented the Sod1p activity in the sod2D cells, but to a lesser extent (Fig. C.15c), apparently insufficient to neutralize the toxic effect that PQ had on the sod2D cells (Fig. C.15b). The SOD activity of the same extracts that were subjected to the in gel assay was determined spectrophotometrically at 560 nm by monitoring the capacity of the extracts to inhibit the superoxide-related reduction of NBT. The extracts isolated from sod1D cells treated with [CuCl(acac)(tmed)] exhibited a remarkably high SOD-like activity which surpassed the activity of the extracts isolated from sod2D cells grown under the same conditions (Fig. C.15d). This observation prompted the possibility that in the absence of native Sod1p, [CuCl(acac)(tmed)] acted as a potent superoxide scavenger within the yeast cells. Nevertheless, when functional Sod1p was present within the cell, the compound seemed to act on the protein, directly or indirectly, by enhancing its activity.

In this case, the intrinsic SOD-like activity of the compound was difficult to assess, since we could not discriminate between the activity of Sod1p and that of the compound in the total SOD activity of the cell extracts determined spectrophotometrically (Fig. C.15d). Nevertheless, it was obvious that the [CuCl(acac)(tmed)] complex had SOD-like activity within the sod1D cells and it strongly activated Sod1p when the latter was present in the cell. This was clearly detectable in the wild-type cells, and to a lesser extent in the sod2D cells, a tendency that was reproducible and was seen both in gel and in spectrophotometric measurements of the same cell extracts (Fig. C.15c, d). This observation suggested the possibility that [CuCl(acac)(tmed)] has SOD-mimetic activity only in the absence of native Sod1p, otherwise acting as a modulator of Sod1p activity.

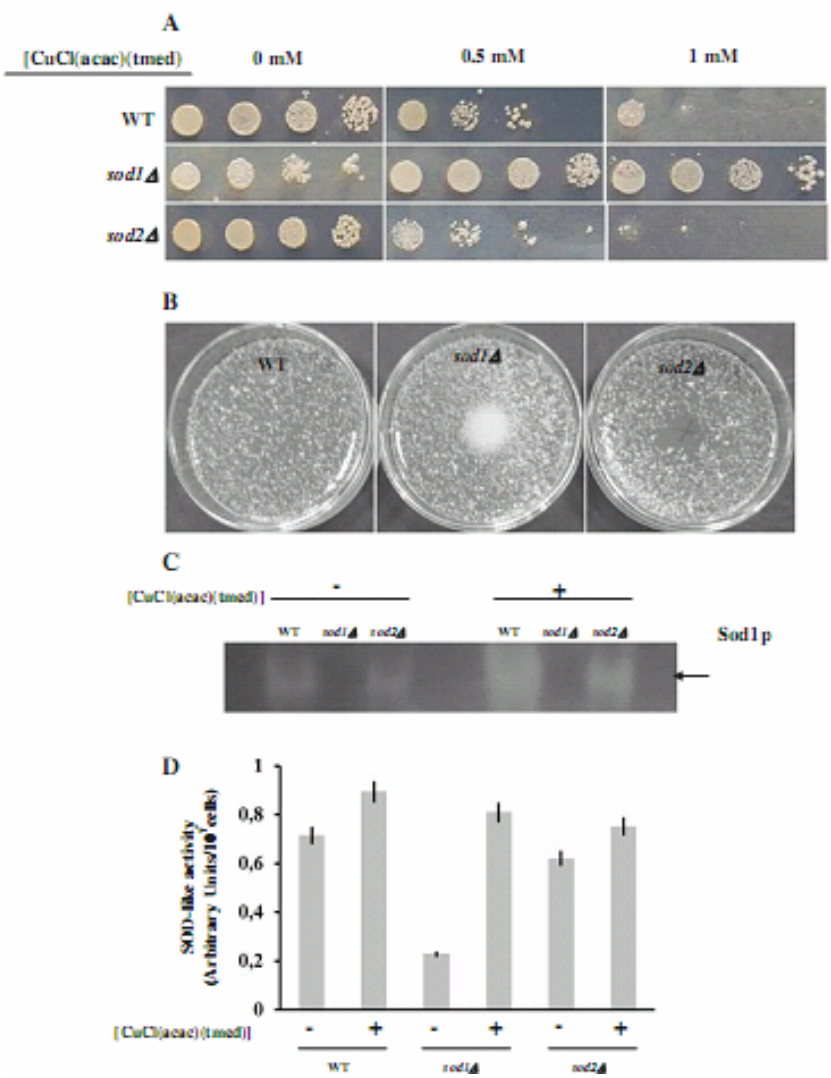


Fig. 3 Effect of [CuCl(acac)(tmed)] on cell growth and SOD activity. a The compound improved the growth of sod1D cells but not that of wild-type and sod2D cells. b [CuCl(acac)(tmed)] abolishes the toxicity of the O21--generating drug toward sod1D cells, but not toward sod2D cells. c SOD activity in yeast cells.

Nevertheless, [CuCl(acac)(tmed)] seems to require functional Ccs1p to exert its modulating activity on Sod1p, but its role is not simply to provide copper ions to Ccs1p through dissociation. This was also supported by the in gel assay of Sod1p activity detected in the extracts obtained from wild-type and ccs1D cells, which revealed that the compound had little effect on Sod1p activation in the absence of Ccs1p, but nevertheless it activated Sod1p more strongly than the simple copper salts.

This observation suggested the possibility that [CuCl(acac)(tmed)] may activate Sod1p not by delivering copper ions to Ccs1p, but by changing the oxidative status of the environment. This idea was strongly supported by the observation that ascorbate abolished the Sod1p activation induced by [CuCl(acac)(tmed)]. It is known that in addition to delivering copper ions to apo-Sod1p, Ccs1p also facilitates the formation of the intramolecular disulfide bond necessary for enzymatic activity in the presence of molecular oxygen. It is possible that the SOD-like activity of [CuCl(acac)(tmed)] provides

local augmentation of O_2 and/or H_2O_2, activating Sod1p by changing its sulfide status. In support of this idea, the Sod1p activation was also detected when the medium was supplemented with exogenous H_2O_2. In contrast, Sod1p activation was inhibited by addition of nontoxic Cd^{2+}, a potent interactor with the sulfhydryl groups.

The hypothesis that [CuCl(acac)(tmed)] activates Sod1p by oxidative change prompted us to investigate the possibility that the compound might act differently on mutants with different oxidative statuses. To test this assumption, we checked the effect of the compound on the growth of several knockout mutants with various defects in the defense against the oxidative stress. A first screen selected the mutants which exhibited altered tolerance to [CuCl(acac)(tmed)] when compared with the wild type. Apart from sod1D, only the skn7D and ahp1D knockout mutants exhibited increased tolerance to [CuCl(acac)(tmed)]; tsa1D and hyr1D were the least tolerant. Nevertheless, this apparent toxicity was not conclusive, as the two mutants also exhibited slow growth in nonsupplemented media. Supplementing the growth media with equimolar concentrations of Cu^{2+} or tmed did not have the same effect as the complex itself. Surprisingly, the mutants which exhibited altered tolerance to [CuCl(acac)(tmed)] also showed a different level of Sod1p activity under standard growth conditions, suggesting that alterations in the oxidative state modulate Sod1p activity.

We next focused on skn7D, as it was the only mutant tested which exhibited lysine auxotrophy that could be alleviated by the compound. Skn7p is a transcription factor that regulates the response to oxidative stress along with the more important factor Yap1p[33]. Unlike skn7D cells, the growth of yap1D cells was slightly impaired by the Cu(II) complex. The effect of [CuCl(acac)(tmed)] on the Sod1p activity was further assayed in skn7D cells in comparison with yap1D cells and the tolerant mutant ahp1D. When cells were grown in normal YPD medium, the Sod1p activity was very low in both skn7D and yap1D cells. In the skn7D mutant, however, the very low basal Sod1p activity was significantly enhanced by [CuCl(acac)(tmed)], whereas in the yap1D mutant, the enhancement was more modest. In contrast, Sod1p activity was constantly high in ahp1D cells, both in the absence and in the presence of the compound. The overall activity determined spectrophotometrically in the extracts obtained from the yap1D cells treated with the compound was similar to that of the extracts obtained from the skn7D cells, probably owing to the intrinsic SOD-like activity of the compound which was present in the extract.

Interestingly, the Sod1p activity was constitutively high in the ahp1D cells and was not significantly altered by the compound. Nevertheless, the overall SOD activity determined spectrophotometrically in the extracts isolated from the ahp1D cells treated with the compound was slightly higher than that of nontreated cells, suggesting that in the presence of highly active Sod1p, the compound had the ability to exert its SOD-like activity. Ahp1p is a thioredoxin peroxidase which localizes predominantly in the cytosol, and is involved in scavenging of alkyl hydroxyperoxides,

[33] Mulford KE, Fassler JS, *Eukaryot Cell*, **2011**, 10, 761

especially in the stationary phase[34]. In ahp1D cells, the altered oxidative state of the cell may be responsible for the Sod1p activation. This would account for the increased tolerance of this mutant to superoxide-generating agents, such as PQ and menadione.

Conclusions

With the large number of diseases related to altered levels of superoxide ions and owing to difficulties encountered when using purified SOD, identification of small molecules with SOD-mimetic activity or with SOD-modulating action has attracted much attention. As removal of superoxide is redox-based, it was natural that the search for SOD mimics be concentrated primarily on metal complexes that possess a redox-active metal site and rich coordination chemistry. In this respect, Cu(II) coordination compounds are intensively studied. We found that [CuCl(acac)(tmed)], a Cu(II) complex with mixed ligands, exhibited SOD-like activity, but also SOD-modulating activity in yeast cells.

[34] Lee J, Spector D, Godon C, Labarre J, Toledano MB, *J Biol Chem* , **1999**, 274, 4537 b) Park SG, Cha M-K, Jeong W, Kim I-H, *J Biol Chem*, **2000**, 275, 5723

Articles and conferinces:

1. Ioana Dumitru, Cristian D. Ene, Codruța C. Paraschivescu, Augustin M. Ofițeru, Augustin M. Mădălan, Baciu Ion, Ileana C. Fărcăşanu, *Journal of Biological Inorganic Chemistry,* 17 (6), **2012**, 961-974
2. Cristian Dobrotă, Domenico Fasci, Niculina D. Hădade, Gheorghe-Doru Roiban, Cristina Pop, Veronika M. Meier, Ioana Dumitru, Mihaela Matache, Guy S. Salvesen, Daniel P. Funeriu, *ChemBioChem*, 13(1), **2011**, 80-84
3. Ioana Dumitru, *Synlett.*, 3, **2011**, 0432-0433
4. Radu Mitrica, Ioana Dumitru, Lavinia L. Ruta, Ileana C. Farcasanu, *Molecules*, **2012**, 17 (9), 10355-10369
5. *Design and synthesis of activity-based probes for the SENPs enzyme family*, Dobrotă Cristian, Bogdan Niculina, Dumitru Ioana, Funeriu Daniel, 2nd EuCheMS Chemistry, Congress, poster, *Torino*, Italia, **2008**
6. *The use of chemical protein functionalization technique for the synthesis of new protein based polymers,* Ioana Dumitru, Mihaela Matache, Niculina D. Bogdan, Ion Baciu, Daniel P. Funeriu, 2nd International Colloquim on "Physics of Materials", poster, *Bucuresti,* **2010**
7. Comunicări orale la Sesiunile de Comunicări Ştiinţifice Studenţeşti ale Facultăţii de Chimie, Universitatea din Bucureşti, în anii 2009 (premiul II), 2010 (III) şi 2011 (menţiune)

Other articles:

1. *Overexpression of the PHO84 gene causes heavy metal accumulation and induces Ire1p-dependent unfolded protein response (UPR) in Saccharomyces cervisiae cells*, Augustin Ofiteru, Lavinia Ruta, Codruta Rotaru, Ioana Dumitru, Cristian Ene, Aurora Neagoe, Ileana Farcasanu, *Applied Microbiology and Biotechnology,* 94 (2)*,* **2012***,* 425-435
2. *Exogenous oxidative stress induces Ca2+ release in the yeast Saccharomyces cerevisiae cells*, Popa Claudia; Dumitru, Ioana; Ruta, Lavinia; Danet, Andrei; Farcasanu, Ileana, *FEBS*, 227 (19), **2010**, 4027-4038
3. *Expedient access to fused quinoxalines via Dess-Martin periodinane-mediated cascade cyclizations of unsymmetrical homoallylic phenylenediamides derivates*, Cristian Dobrota, Jonathan Graeupner, Ioana Dumitru, Mihaela Matache, Codruta C. Paraschivescu, *Tetrahedron Letters,* 51 (9), **2010**, 1262-1264
4. *Protein-Inorganic Array Construction: Design and Synthesis of the Building Blocks*, Niculina Bogdan, Mihaela Matache, Veronika Meier, Cristian Dobrota, Ioana Dumitru, Gheorghe Roiban, Daniel Funeriu, *Chemistry - A European Journal,* 16 (7), **2010**, 2170-2180

5. *Synthesis of fused dihydro-pyrimido[4,3-d]coumarins using Biginelli multicomponent reaction as key step*, Mihaela Matache, Cristian Dobrotă, Niculina Bogdan, Ioana Dumitru, Lavinia Ruță, Codruța Paraschivescu, Ileana Fărcăsanu, Ion Baciu, Daniel Funeriu, *Tetrahedron*, 65 (31), **2009**, 5949-5957
6. *Convenient preparation of unsymmetrical 2,5-disubstituted 1,3,4-oxadiazoles promoted by Dess-Martin reagent*, Cristian Dobrotă,Codruța Paraschivescu, Ioana Dumitru, Mihaela Matache, Ion Baciu, Lavinia Ruță, *Tetrahedron Letters,* 50 (17), **2009**, 1886-1888