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TESI DI DOTTORATO

PROGETTAZIONE, SINTESI E CARATTERIZZAZIONE BIOCHIMICA DI INIBITORI DI PROTEIN CHINASI

DESIGN, SYNTHESIS AND BIOCHEMICAL CHARACTERIZATION OF INHIBITORS OF PROTEIN KINASES

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To my beautiful grandmother Eufemia

"According to the laws of aerodynamics, the bumblebee should not be able to fly because the weight of his body is disproportioned to the surface of his wings. But the bumblebee doesn't know that and goes on flying anyway!"

Secondo i più eminenti scienziati il calabrone non potrebbe volare perché il peso del suo corpo è sproporzionato alla portata delle sue ali…….ma il calabrone non lo sa e vola.

Index

Amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophane
Y	Tyr	Tyrosine
Sp		Phosphoserine
Tp		Phosphothreonine
Yp		Phosphotyrosine
X		whatever amino acid

Abbreviations

AA	amino acid
abs	absolute
ADP	adenosine diphosphate
ATP	adenosine triphosphate
cAMP	3’-5’-cyclic adenosine monophosphate
cGMP	3’-5’-cyclic guanosine monophosphate
CK1	casein kinase 1
CK2	casein kinase 2
GTP	guanosine triphosphate
PKA	protein kinase A
PKC	protein kinase C
rhCK2α	recombinant subunit α of human CK2
rmCK2α	recombinant subunit α of CK2 from *Zhea Mays*

Riassunto

In questo elaborato viene descritta l'identificazione e la caratterizzazione di alcuni nuovi potenti inibitori di CK1 e di CK2.

Sono stati sviluppati quattro distinti sotto progetti.

Con l'obiettivo di pervenire a una semplificazione strutturale, nella prima parte sono stati sintetizzati alcuni derivati dell'acido ellagico, un inibitore di CK2 da noi recentemente identificato la cui struttura ricorda quella di due cumarine condensate.

Particolare attenzione è stata rivolta all'urolitina A, uno dei metaboliti bio-attivi dell'acido ellagico che pure è risultata efficace contro la CK2 specialmente dopo l'introduzione di un atomo di bromo o di un nitro gruppo in posizione 4 che riportano le costanti di inibizione nel basso nano molare. Nella seconda parte sono stati studiati una serie di derivati poliiodinati del benzoimidazolo sia in relazione alla loro efficacia che alla selettività. I risultati mostrano che i 4,5,6,7-tetraiodobenzimidazoli sono in genere di un ordine di grandezza più potenti rispetto ai loro analoghi tetrabromoderivati. Dati ottenuti con tecniche di modellistica molecolare mostrano che la struttura del tetraiodobenzimidazolo si colloca meglio, all'interno della tasca nucleotidica della chinasi riempiendola più efficacemente, rispetto ai corrispondenti tetrabromo e tetracloro derivati.

Nella terza parte, si è studiata l'efficacia e la selettività di un nuovo antrachinone identificato con un approccio di virtual screening, la quinalizarina, che si è rivelato più potente e selettivo della emodina. Oltre a un potere inibitorio ottimale, con una K_I circa uguale a 50 nM, particolarmente interessante è l'abilità della chinalizarina di discriminare tra CK2 e un numero di chinasi, tra cui DYRK1a, PIM 1,2, e 3, HIPK2, MNK1, ERK8 e PKD1, che di solito tendono ad essere inibite altrettanto efficacemente dai più comuni inibitori di CK2.

Infine è stato svolto un lavoro anche sulla protein chinasi CK1 con l'obiettivo di individuare nuovi composti capaci di inibire selettivamente le diverse isoforme di questa protein chinasi.

Abbiamo così individuato due nuovi antrachinoni come nuovi potenziali inibitori di CK1δ. Con saggi di attività sulle diverse isoforme di CK1, così come su un discreto numero di altre protein chinasi, si è dimostrata l'alta selettività nei confronti dell'isoforma δ di CK1 e un potere inibitorio simile ai migliori inibitori di CK1 presenti in commercio.

Abstract

The work described in this thesis is dealing with the identification and characterization of some new potent inhibitors of protein kinases CK1 and CK2.

Four distinct sub-projects have been carried out.

In the first part some derivatives of ellagic acid, a powerful recently discovered CK2 inhibitor, have been synthesized with the aim of simplifying the ellagic acid structure composed of two condensed coumarins. Particular attention was dedicated to urolithin A, a bio-active metabolite of ellagic acid, proved to be also effective on CK2 especially after the introduction of a bromine or of a nitro group at position 4, leading to a very high efficiency in the low nanomolar range.

In the second part a series of polyiodinated benzimidazoles has been investigated for both inhibitory power and selectivity toward protein kinase CK2. The results show that 4,5,6,7-tetraiodobenzimidazoles display in general one order of magnitude lower IC_{50} values as compared with their tetrabrominated analogs. Molecular modeling supports the experimental data showing that the presence of iodine atoms as substituents of the benzene moiety leads to a better filling of the nucleotide binding pocket of the kinase.

The third sub-project, investigated the potency and selectivity of a newly identified anthraquinone, quinalizarin, as an inhibitor of CK2 proved to be more potent and selective than emodin. Besides a very good efficiency, with a K_I value of approx. 50 nM, especially remarkable is the ability of quinalizarin to discriminate between CK2 and a number of other kinases, notably DYRK1a, PIM 1,2, e 3, HIPK2, MNK1, ERK8 and PKD1 which conversely tend to be often inhibited as drastically as CK2 by other known CK2 inhibitors.

Finally protein kinase CK1 was also taken in consideration with the aim of identifying new compounds able to selectively inhibit the different isoforms of this protein kinase.

In this case we have identified two anthraquinones proved to be among the most potent and selective inhibitors of the CK1δ isoform. Activity assays performed on different isoforms of CK1 as well as on a number of other protein kinases demonstrated high selectivity toward isoform δ and a potency comparable to that of other commercially available CK1 inhibitors.

Chapter 1

The Protein Phosphorylation

1.1 GENERAL

Protein kinases are a class of enzymes which are implicated in a chemical reaction in which the terminal phosphate group of one molecule of ATP or, more rarely of GTP is transferred to a protein molecule that acts as a substrate. The opposite reaction is ensured by the presence of other enzymes, the phosphatases, catalyzing the reverse reaction. The kinases possess therefore a double anchoring site, one for the binding of the phosphate donor and one for the binding of the phosphate acceptor molecule. These substrates bind to the protein kinases simultaneously in specific “pockets” to form the catalytic ternary complex. The transfer of the phosphate group to the protein substrate occurs in a single step, without the temporary "parking" of the phosphate on the enzyme.

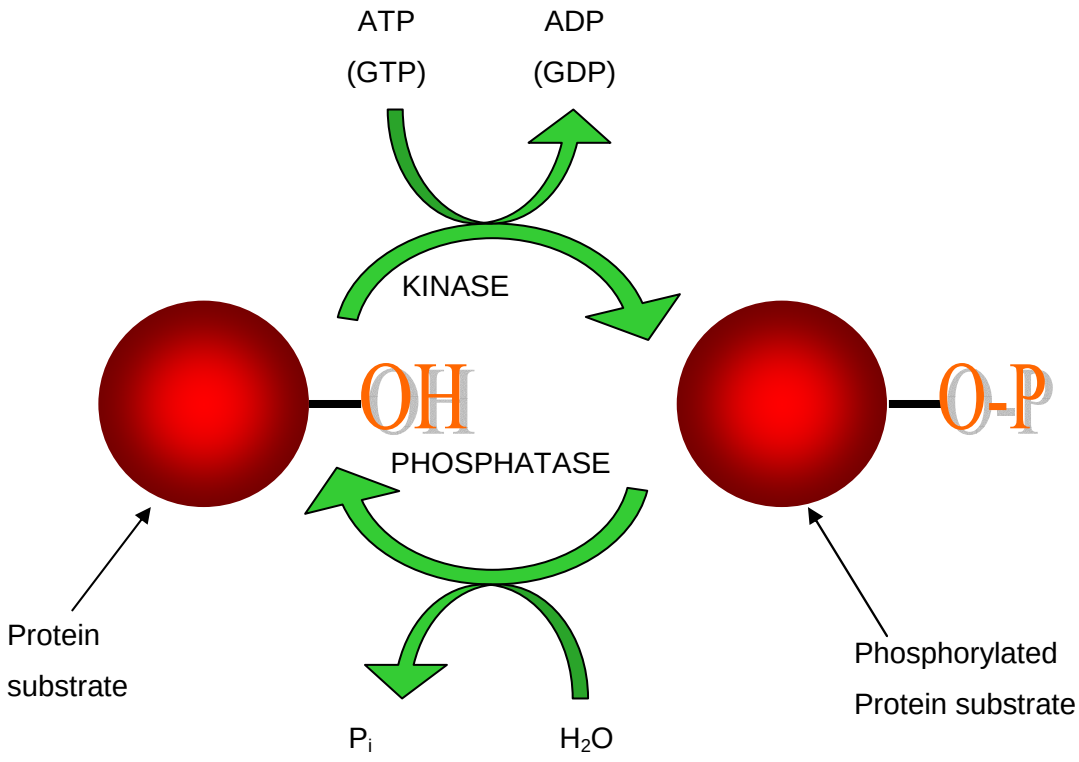


Figure 1: Enzymatic activity of a protein kinase

The protein phosphorylation plays an essential role in regulating many cellular functions common to all eukaryotes, such as DNA replication, gene transcription, control of cell cycle, intracellular transport of protein and energy metabolism. In more

advanced organisms, in addition, protein phosphorylation appears to be also required for specialized functions such as differentiation and inter- and intra-cellular trafficking.
Special consideration must be deserved in the case of viruses: these organisms usually do not have their "own" protein kinases. In most cases, the virus "exploits" the host's kinases to phosphorylate viral components required for the progression of the infection.
The phosphorylation within the cell is generally controlled by extracellular signals such as hormones, growth factors, neurotransmitters and antigens that bind to specific membrane receptors. These receptors may regulate the activity of protein kinases and phosphatases acting through some changes in the concentration of cytoplasmic second messengers such as cAMP, which regulates the protein kinase A (PKA)[1] or the Ca^{2+}/diacylglycerol, which activates the protein kinase C (PKC)[2]; others, such as, for example, receptors with tyrosine kinase activity, directly activate either a kinase component associated with themselves, without the need of second messengers , or a kinase domain located in their cytoplasmic region.

In *Saccharomyces cerevisiae* the genes encoding for the kinases represent a substantial part of the whole genome: 121 out of 6144 genes in fact of yeast (about 2%) encode for protein kinases.
Similarly in *Drosophila melanogaster* 319 out of 13,338 genes encode for protein kinases and in the nematode worm *Caenorhabditis elegans* there are about 437 out of the 18,366 genes that encode these proteins. According to a rough estimate, about one third of mammalian proteins contain a phosphate group covalently linked.

By extrapolation from the genomes of yeast and nematode, it was expected that there were no fewer than 1000 protein kinases and 500 protein phosphatases involved in phosphorylation of human proteins. Instead, everything was coded in about 550 kinases in the human genome, grouped into over 57 families corresponding to 2% of the genome. Only a small part of the complex functional interactions of these proteins has been discovered[3]. Numerous studies have underlined the importance of the activity of kinases and phosphatases to control cellular function; in fact an imbalance at this level has a role to increase diseases. More than 400 human diseases, including diabetes, rheumatoid arthritis, many cancers (leukemias and lymphomas) and viral diseases are caused by abnormal levels of phosphorylation.

A protein kinase is characterized by a bilobal architecture that consists of a smaller upper lobe mainly cointaining β-sheet rich domains and a lower lobe of larger size rich in α-helices. Between the two lobes there is a deep inflexion of the protein surface harbouring the ATP binding site. A particular functional role in the phosphate transfer reaction has been assigned to the glycine rich loop, to the catalytic loop and to the activation loop but also other subdomains were implicated, for instance, in the coordination of Mg^{2+} ions. A typical example of a protein kinase structure is given by the protein kinase A (PKA).

1.2 CLASSIFICATION OF PROTEIN KINASES

1.2.1 "BIOCHEMICAL" CLASSIFICATION

The biochemical classification of protein kinases is based on their amino acid residue that can accept the phosphate group[4] and comprises three main families:

- *Ser/Thr-specific protein kinases*: utilize the alcoholic group of a serine and/or a threonine as acceptor of the phosphate group;

- *Tyr-specific protein kinases*: catalyze the phosphorylation of the phenolic group of the tyrosine;

- *Dual-specificity protein kinases*: able to phosphorylate both Ser/Thr and Tyr residues

Besides the above mentioned classes of kinases, altogether accounting for the majority of mammalian members of these enzymes, there are also:

- *His protein kinases*: this group of enzymes catalyze the phosphorylation of a residue of histidine at position 1 or 3, but also, in some cases the guanidyl group of the arginine and the amino group of the lysine side chain;

- *Cys protein kinases*: utilize thiolic group of a cysteine as acceptor of phosphate group;

- *Glu and Asp protein kinases*: catalyze the phosphorylation of the carboxylic group of the side chain of glutamic and aspartic acid residues

As already outlined the Ser/Thr- and Tyr-specific protein kinases represent a quite large family of enzymes and have been characterized much better than the others.
From a more functional point of view it is possible, however, to highlight some important sub-families of protein kinases which are commonly used to indicate similar mechanism of action:

- *protein kinases regulated by cyclic nucleotides as cAMP or cGMP*: to this group belongs the well known cAMP-dependent protein kinase (PKA), one of the first protein kinases to be studied[5] and the first kinase known in its tridimensional structure[6];

- *protein kinases regulated by diacylglycerol (Ca^{2+}/phospholipids)*: their activity is regulated by diacylglycerol and by some lipidic components. For example PKC (which is known in more than 10 isoforms)[7];

- *S6 kinase family*: for example the kinase involved in the phosphorylation of ribosomial S6 protein[8];

- *protein kinases dependent on cyclins (CDK)*: a group of Ser/Thr eukaryotic protein kinases[9]; the association is occurring upon the binding with a cyclin and through a series of phosphorylation/dephosphorylation events at conserved sites producing the activation/deactivation of the enzyme and regulating the transition through the different phases of cell cycle;

- *independent protein kinases*: they include Ser/Thr protein kinases able to escape all classifications for being constitutively active. Their regulation is actually poorly understood and hardly consistent with the mechanism of activation of the majority of the other protein kinases which are usually silent and become activated upon the intervention of second messengers or other kinases. Protein kinase CK2 is belonging to this group.

1.2.2 "PHYLOGENETIC" CLASSIFICATION

In this classification, the eukaryotic protein kinases differ on the basis of structural and functional features. It is possible from this point of view to divide the family of protein kinases into 5 groups on the basis of the alignment of their catalytic domains[10]; in fact it is conceivable that kinases displaying similar catalytic domains are encoded by genes recently diverged in the evolution.
The five groups are the following:

1. **"AGC" group (PKA, PKG, PKC)**: this group mainly includes Ser/Thr-specific protein kinases exemplified by PKA and PKG (both cyclic nucleotides dependent) and PKC (Ca^{2+}/phospholipids dependent);

2. **"CAMK" group**: this group includes the family of protein kinases regulated by Ca^{2+} and calmodulin. As already mentioned, in some cases calmodulin represents one of the enzyme's subunit (as in the case of phosphorylase kinase), in others calmodulin is bound to specific domains of the kinase;

3. **"CGMC" group (CDK, GSK3, MAPK, CK2)**: here we can found an heterogeneous group of kinases including, besides CDKs, other apparently unrelated families. As far as the site specificity is concerned, most of these kinases are "proline-directed". CK2, however, is one of the few examples of kinases preferring acidic substrates;

4. **“PTK” group**: this is the numerous family of tyrosine protein kinases; it is divided into 30 subfamilies, 10 of which are classified as “non-receptor” and 20 as “receptor” tyrosine kinases;

5. **“Various” group**: in this group there are all the protein kinases not clearly belonging to other groups because they are recently discovered or their physiological substrates are poorly understood. This group still includes the family of CK1 kinases for which recent information in literature supports an important role in the Wnt signalling[11] and the dual protein kinase MEK.

1.3 IMPORTANCE OF PROTEIN KINASES

Kinases are proteins mostly involved in signal trasduction. They modify the activity of their protein substrates controlling a lot of cellular processes including, among others, metabolism, transcription, cell cycle progression, cytoskeleton rearrangement and cell motility, apoptosis and differentiation. Protein phosphorylation is essential also in the intracellular communication, in the physiological response, in homeostasis and in immunity and nervous system function. A deregulation of protein kinases (usually an hyperactivation) is able to cause diseases. For this reason it is important the design and production of specific inhibitors of protein kinases.

Disease	Involved kinases	Disease	Involved kinases
Inflammation	ERK, P38, JNK	Essential hypertension	ERK, P38
Insulin-dependent diabetes	GSK3, PKC	Pulmonary hypertension	ALK1
Insulin-independent diabetes	GSK3, PKC	Cardiovascular disease	ERK
Obesity	PKA	Down's syndrome	DYRK1a
Alzheimer	GSK3, PKC, ERK, CDK5, CK1δ	Craniosynostosis	FGFR
Parkinson	JNK, DYRK1a	Infectious and parasitical disease	CK2, CK1
Schizophrenia and depression	CAMKII	Cancer	Aurora, CK1δ, CK2, RTK, NRTK

Table1: Some of common diseases in which protein kinases are clearly implicated

Most of inhibitors of protein kinases behave as ATP-mimetics because they compete with the nucleotide phosphodonor substrate in the catalytic site of the enzyme.

They are however structurally different from ATP but nevertheless they are able to interact with specific residues localized in the close proximity of ATP binding site, preventing the binding of ATP and the phosphate transfer to the protein substrate.

Chapter 2

Casein Kinases

2.1 HISTORICAL CONSIDERATIONS

CK2 is one of the first protein kinases to have been detected, and perhaps it may be the very first known kinase[12]. The first casein kinase activity was identified in 1954 by Burnett and Kennedy[13] through experiments conducted on rat liver using casein as substrate phosphorylation. Later it was demonstrated that the same activity can be detected in many other tissues. This activity was attributed to an enzyme that was described as "casein kinase" or "phosvitin kinase", thereby reflecting its preference for acidic substrates, as model substrates in vitro, including specifically the casein of milk and the phosvitin of yolk, two abundant phosphoproteins and relatively easy to obtain. Only in 1969 it was discovered that the enzyme activity was due to two distinct enzymes[14], which were called CASEIN KINASE 1 and CASEIN KINASE 2 (today known as CK1 and CK2).

If the casein, but also the phosvitin, are ideal substrates to test the activity of casein kinases, they are not physiological substrates. Casein is the only true physiological substrate of one of the members of casein kinases, the so-called G-CK[15,16] committed with the phosphorylation of casein within the lactating mammary gland.

Paradoxical is the fact that the protein kinase CK2, which for nearly two decades since its discovery was left an orphan of its physiological substrates, today is probably the most pleiotropic protein kinase present in eukaryotic organisms[17]. Today there are more than 300 protein substrates for CK2 and their number increases continuously. These substrates are involved in all areas of cell regulation. Despite many efforts and the vast amount of information gathered, is still unclear the regulatory mechanism of this enzyme, but from a variety of coincident observation it is possible to assume that this kinase plays a role in signal transduction pathways, gene expression, proliferation[17,18,19,20,21,22,23]. Also about CK1 the mechanism(s) of regulation are poorly understood, while the mechanisms of G-CK are still fully unknown.

2.2 CLASSIFICATION OF CASEIN KINASES

To the family of casein kinases belong two classes of enzymes:

- **“casein kinase” of mammary gland**: it is a tissue specific casein kinase that is physiologically responsible to phosphorylate the protein fraction of the milk, in which also the casein, that is its natural substrate, is present. This protein fraction is newly synthesized in the Golgi apparatus and is subsequently ejected by exocytosis in collector ducts of the mammary gland. This is the reason of the definition of G-CK (Golgi Casein Kinase). It was later demonstrated that the Golgi apparatus of the liver, spleen, and, to a lesser extent, of kidney and brain of rats, contains a protein kinase activity biochemically indistinguishable from the G-CK of the mammary gland, but whose physiological role, however, is still unclear[24].

- **The ubiquitous protein kinases “casein kinase 1 and 2” (CK1 and CK2)**: their physiological substrates are represented by several enzymes and proteins, involved in numerous biological functions. The term "casein kinase", as mentioned above, is justified by the fact that they show a strong preference towards acidic substrates such as casein and phosvitin, as compared with the commonly used basic substrates such as histones and protamine. In order to avoid, however, possible confusion with the true "casein kinase" of the mammary gland, is was recently proposed to name these two types of enzymes “**protein kinase CK1 and protein kinase CK2**”. The numbering in the name of these two kinases is referred to the order of elution from a column of DEAE-cellulose, in the early stages of purification. Unlike the G-CK, which is located at the level of the Golgi apparatus and is absent in the rest of the cell, CK2 and CK1 are hardly detectable in this cellular compartment[24]. CK2 is present in several subcellular fractions, especially in the nucleus where it reaches its highest concentration[25,26].

It is possible to observe that:

1. **CK1** and **CK2** are different in structure, specificity, response to effectors and show a different binding to the phosphocellulose resin[27]. This latter feature is exploited to separate the two kinases in cellular extracts; CK2 requires a greater ionic strength to be eluted from phosphocellulose with respect to CK1.

2. **CK2** has a primary structure with considerable degree of conservation in evolutionary distant organisms and it is not inhibited by staurosporine, one of the most potent inhibitor of protein kinases.

3. **CK1** and **G-CK** are able to use only ATP as phosphate donor, while **CK2** can use almost with the same efficiency both ATP and GTP as phosphate donors. Also **CK2**, as the other kinases, requires the presence of divalent cations (especially magnesium, but also manganese and cobalt) and it was noted that, in the presence of magnesium ions, the affinity is higher for ATP as compared to GTP, whereas the opposite is true in the presence of manganese ions.

4. Another aspect that distinguishes **CK2** from the other two classes of casein kinases, is its heterotetrameric structure, a feature not very common among the protein kinases. Moreover, the three forms of casein kinases display a quite distinct site specificity as summarized in the table below (Table 2).

CHARACTERISTICS	CK2	CK1	G-CK
Tissue distribution	ubiquitary	ubiquitary	mammary gland
Physiological substrate	Various proteins	Various proteins	casein and others
Molecular Weight (kDa)	120-150	25-60	400
Structure	oligomer (heterotetramer) α2β2 / αα′β2 / α′2β2	monomer	monomer
Subunit	α, α′, β	-	-
Molecular Weight (kDa)	α =36-42;α′ =36-42; β=26	-	-
Phosphate donor	ATP/GTP	ATP	ATP
Phosphorylated residues	Ser/Thr	Ser	Ser
Consensus sequence	S/T-X-X-E/D/Sp/Yp**	Sp*X-X-S/T	S-X-E/Sp

Table2: General characteristics of “casein kinases” CK1, CK2 and G-CK; *Phosphoserine;** In the case of CK2 glutamic acid and aspartic acid can be replaced by phosphoserine and phosphotyrosine as specificity determinants.

2.3 PROTEIN KINASE CK1

2.3.1 DISTRIBUTION

The protein kinase CK1, or casein kinase 1, is a monomer present in all eukaryotes from yeast to human, in all tissues and cellular compartments and localyzed in nucleus and in cytoplasm. Sometimes is associated, in a stable way, to the cellular membrane or to proteins of cytoskeleton.

CK1 has been involved in numerous cellular processes, for example in the repair of damaged DNA by radiations[28], in the regulation of the circadian rhythm in *Drosophila*[29] and in human[30], in the cell division[31] and in neurotransmission[32].

CK1 is a Ser/Thr kinase; it has been isolated and characterized and its distribution has been thoroughly studied. From the biochemical and functional point of view, recent approaches have led to a number of interesting, albeit sometimes contradictory, observations and today the existence of multiple isoforms, each one with different biochemical properties, is universally accepted.

2.3.2 STRUCTURAL ASPECTS :

THE ISOFORMS OF CK1 OF SUPERIOR EUKARYOTES

The first codifying cDNA, isoform of bovine CK1 has been isolated by Rowles in 1991. Since that discovery seven isoforms of CK1 of mammals have been cloned and characterized, that are: α, β, γ1, γ2, γ3, δ and ε. These isoforms contain a number of domains which are common to all other Ser/Thr protein kinases, with the exception of the motif Asp-Pro-Glu (APE) of the domain VIII[33]. All these isoforms contain a sequence of nuclear localization signal (NLS).

The characteristics of the above mentioned isoforms are:

CK1α

The molecular weight of this isoform is 37500 Da, it is ubiquitously expressed in all tissues and in all cellular compartments[34].

In addition to the catalytic domain CK1α has a short C-terminal sequence comparable to the sequence of the other members of the family of CK1.

Its human gene has been mapped in the human chromosomal locus 13ql3 and it has been connected to chronic lymphoproliferation[35].

CK1β

The molecular weight of this isoform is 39000 Da and its primary structure is 79% similar to CK1α. Its carboxy terminal sequence contains an extension of twelve amino acids similar to the sequence present in CK1α'', a splicing isoform of CK1α[36]. Some evidence suggests that its mRNA could be present in other tissues, besides bovine brain, from which the cDNA of CK1β was originally isolated[37].

CK1γ

There are 3 forms of CK1γ genetically distinct. The molecular weights of CK1γ1, CK1γ2 and CK1γ3 are 43000 Da, 45000 Da and 49700 Da, respectively[37] and all the three isoforms are more than 51% similar to the other members of the CK1 family. Outside the catalytic domain they possess C-terminal extensions (about 65-123 amino acids) and an extra amino terminal domain of 26-29 amino acids. The mRNA of γ1 and γ2 isoforms have been found only in testis, while the mRNA of isoform γ3 is present in all tissues except spleen and heart. The gene of isoform γ1 has been mapped in locus 17q25.2-q25.3.

CK1δ

This isoform weighs 49100 Da[38] and is 76% similar to CK1α but its catalytic domain also shows more than 52% of similarity with the other members of the family. It also contains a C-terminal extension of 88 amino acids. From experiments of Northern blotting, it is thought that this isoform is expressed primarily in testis. Its gene has been mapped in locus 19pl3.3.

CK1ε

CK1ε has a MW of 47300 Da[35] and it is very similar to CK1δ displaying more than 98% amino acidic identity within the catalytic domain and more than 40% in the C-

terminus. Northern blotting experiments show that this isoform is expressed in a variety of cellular lines, although its tissue distribution is still poorly understood. Its human gene has been mapped in the chromosomal locus 22ql2.3-13.1, a region which is usually missing in familiar and sporadic meningioma tumors.

2.3.3 TRIDIMENSIONAL STRUCTURE

The molecular genetic technology made possible the crystallization of CK1 from yeast[39] and mammals[40].

The achievement of the 3D structure, obtained by X-ray diffraction, highlighted the similarity with other protein kinases. As in the 3D structure of PKA, we can see here:

the N-terminal upper lobe, smaller than the lower and with a large portion of β-sheets;

the C-terminal lobe, composed by α-helices;

the cleft between the two lobes, involved in the binding of ATP and protein substrate;

a “hinge” region joining the two lobes and determining the overall conformation of the kinase.

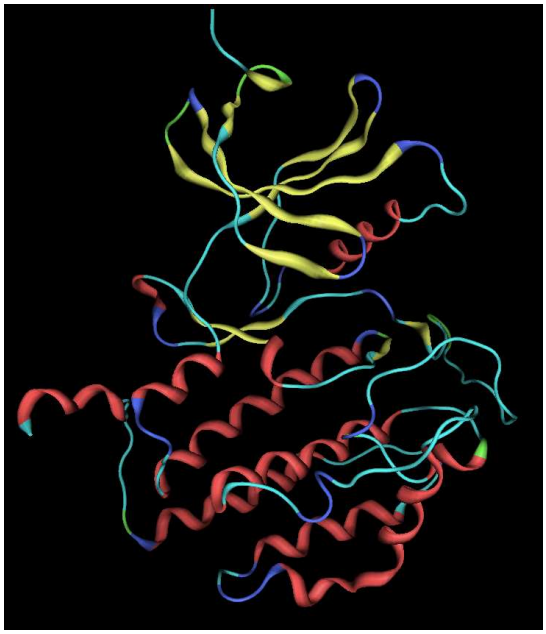


Figure 2. Representation of 3D structure of CK1δ.

Xu et al[39] obtained the first crystal structure of a C-terminal deletion mutant of CK1 from the yeast *S. pombe*. Later the 3D structure of CK1γ was solved in complex with Mg^{2+}-ATP at the resolution of 2.0 Å. The crystal structure of CK1δ has been also obtained and it was found similar to the structure of CK1γ.

It is therefore conceivable that the other components of the CK1 family have a similar structure because of the high degree of conservation (50 to 79% amino acid identity).

2.3.4 SITE SPECIFICITY OF CK1

CK1 was initially classified as a phospho-directed protein kinase, capable of recognizing a phosphorylated residue (usually a phosphoserine) as a specificity determinant. This was based on the observation that its sites of phosphorylation in the casein fractions were invariably preceded by triplets of Serine, constitutively phosphorylated, the dephosphorylation of which prevented the phosphorylation by CK1[41]. This phospho-dependence has been confirmed later with peptide substrates, demonstrating that the crucial minimal determinant is a single phosphoseryl residue located at position n-3, or, less effectively, at position n-4, with respect to the target serine[42,43]. Phosphothreonine (but not phosphotyrosine) can substitute phosphoserine as a positive determinant[44].

Single carboxylic residues proved to be almost ineffective; however, it has been subsequently demonstrated that multiple carboxylic sequences on the N-terminal side of target serine can replace phosphoserine as specificity determinant, as for example in the case of inhibitor-2 of protein phosphatase 1 and in DARPP-32. In fact both these proteins are phosphorylated by CK1 also in the absence of a previous phosphorylation. Moreover, synthetic peptides reproducing the sites of CK1 phosphorylation in the Inhibitor-2 substrate were also phosphorylated albeit with higher K_m values[45].

In summary, therefore, it can be concluded that the consensus sequence for the CK1-mediated phosphorylation is practically specular to the consensus sequence of CK2 (see below).

Sp/Tp – X – X – S/T – B	**B: hydrophobic**
(D/E)n – X – X – S/T – B	**B: hydrophobic**

Recent studies performed by using synthetic peptides provided further demonstration of CK1 consensus sequence, outlining on one hand the negative effect of basic residues located in close proximity to the target serine and, on the other, the positive effect of hydrophobic chains at the C-terminal side. Moreover the 3D structure of CK1 gave some hints about the recognition of the phosphate group by CK1. In fact, a specific binding site for the phosphate was found, thanks to the ability to make complexes with negatively charged moieties (phosphates and tungstates)[39,40].
As far as the CK1 site specificity is concerned, it is important to underline that some isoforms are able to phosphorylate also residues of tyrosine[46]. In particular, the isoforms of yeast Hrr25p, Hhpl e Hhp2 can phosphorylate the co-polymer polyGlu-Tyr (4:1) and self-phosphorylate at tyrosine in vivo and in vitro. Also the isoform CK1α of *Xenopus laevis* is able to phosphorylate the same substrates at tyrosyl residues[47].

2.3.5 SUBSTRATE SPECIFICITY OF CK1

Among the multilple functions of CK1, on the basis of the substrates identified along the years, we can remember:

- Control of metabolic pathways: in the cytosol, CK1 acts in synergism with PKA to phosphorylate and negatively regulate the activity of glycogen synthase[48]. It is also able to regulate the activity of some Ser/Thr protein phosphatases (type 1 and 2). In fact it phosphorylates Ser86 and Ser174 of the Inhibitor-2, a regulatory subunit of protein-phosphatase 1 (PP1)[49];

- Control of transcriptional pathways: CK1 phosphorylates serine 123 of antigen T of SV40, and inhibits its ability to reply the viral DNA and affects cell transformation[50]. CK1 phosphorylates the C-terminal domain of RNA polymerase II, only in association with other kinases[51];

- Control of signal transduction pathways: at the level of cell membrane, CK1 is involved in the signal of TNFα p75 receptor to regulate the production of cytokines, the cell proliferation and the apoptosis in lymphoid cells[52]. The inhibition of CK1 blocked the apoptosis TNFα p75-mediated in vivo. CK1 phosphorylates the muscarinic receptor M3, rodopsine and probably other G protein associated receptors, suggesting that this kinase could be implicated in the desensitization of the receptor[53]. The same kinase phosphorylates an active form of the γ subunit of the insulin receptor[54]. CK1ε was demonstrated to play crucial roles during the events of signal transduction mediated by Wnt[11,55]. CK1 is involved in the regulation of nuclear translocation of the transcription factor NF-AT;

- Control of mechanism of DNA repair: CK1δ and CK1ε are involved in the regulation of p53, a factor involved in the response to the DNA damage[56].The expression of CKIδ is influenced by the level of p53 and by DNA damage, suggesting that a functional interaction between the two molecules can exist;

- Control of neuronal and neuromuscolar processes: it is demonstrated that CK1α is able to regulate the vescicular traffic and the realize of neurotransmitters by small synaptic vesicles[57]. CK1 can also modify cytoskeleton components like spectrin, neurofilaments and the molecule-I of cellular adhesion[58,59].

- Control of cellular cycle: recent studies indicated CK1α for the regulation in vivo of the progression of cell cycle during the mouse development, probably for the subcellular localization of this isoform, which is often associated with cytosolic vesicles, centrosome, mitotic spindle and various nuclear structures.

2.4 THE PROTEIN KINASE CK2

2.4.1 DISTRIBUTION TISSUE-SPECIFIC AND SUBCELLULAR LOCALIZATION OF CK2

Protein kinase CK2 is ubiquitously distributed in eukaryotic organisms, where it most often appears to exist as tetrameric complexes consisting of two catalytic subunits α, α' (with molecular weight respectively of 44 kDa and 38 kDa) and two regulatory subunits β (with molecular weight of 26 kDa).

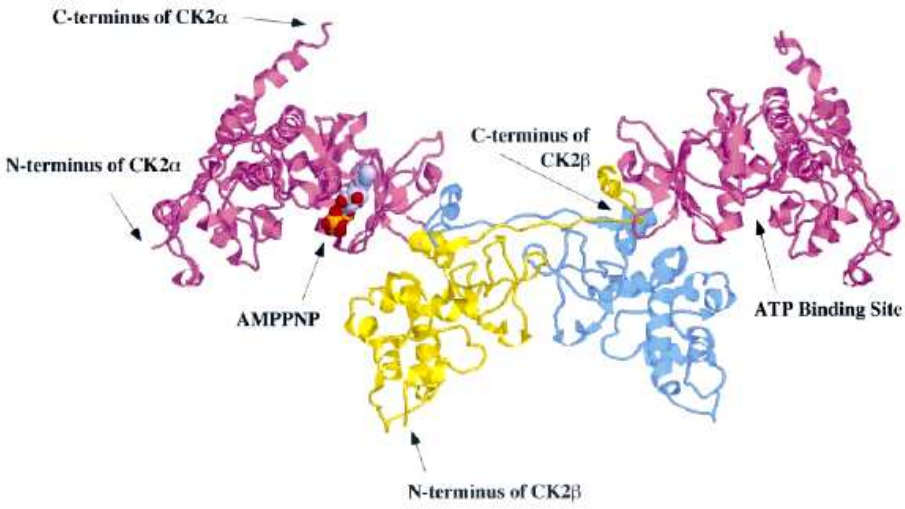


Figure 3: Ribbon diagram illustrating the high-resolution structure of tetrameric CK2[23]

In many organisms, distinct isoforms of the CK2 catalytic subunit have been identified. For example, in human, two catalytic isoforms, CK2α and CK2α', have been well characterized, while also a third isoform, CK2α'', has been identified[60,61]. On the contrary, in human, only a single regulatory subunit, CK2β, has been identified, but multiple forms of CK2β have been identified in other organisms, such as *Saccharomyces cerevisiae*[62]. Several complementary lines of evidence indicate that dimers of CK2β occupy the core of the tetrameric CK2 complexes. In mammalian CK2, tetrameric complexes may contain identical (i.e. two CK2α or two CK2α') or different (i.e. one CK2α and one CK2α') catalytic subunits isoforms[63].

Analysis of tissue distribution of mRNA levels of subunits α, α' and β of CK2 has evidenced that in spleen, brain, ovary and heart, these mRNAs are much more abundant than in kidney and lung. The distribution appears to be tissue-specific. Spleen and heart express high levels of α'-mRNA, whereas the opposite is true for liver, brain and ovary[64].

Further confirmation of the different tissue distribution of CK2 also comes from recent analysis of the levels of α and β subunits during mouse embryogenesis by using in situ hybridization and immunohistochemistry techniques. These studies show that in the early stages of embryo development, CK2 is expressed more in neuroepitelial than in all other tissues[65]. This is also in agreement with the observations of several authors suggesting that increased activity of CK2 can be detected in brain and testes[66].

Another interesting aspect is the lack of correlation, in terms of quantity, between β mRNA and α/α'-mRNA. Although the difference in mRNA level distribution of CK2 subunits does not necessarily reflect the situation at the protein level, the analysis of expression of CK2 in various tissues strongly supported the idea that the subunits may be independently involved in different specific functions[67,68].

According to some authors there would be an almost equal distribution between nucleus and cytoplasm, or even an exclusive presence in the cytoplasm. For others CK2 is a predominantly nuclear enzyme. In a few reports it seems that CK2 is associated with nucleoli, and this is consistent with the fact that nucleolin was found to be one of the best substrates for CK2[69]. Furthermore, this protein kinase might play a significant role in the transmission of regulatory signals within the nucleus.

On the other hand, several authors have suggested that levels of nuclear CK2 are much higher in proliferating cells than in quiescent ones and that there might be a regulated nuclear translocation of this enzyme[19]. It is not yet known whether the subunits migrate from the cytoplasm to the nucleus alone or complexed by proteins. Both subunits contain the “nuclear localization sequence” (NLS) that, in the case of the catalytic subunit, is located at the N-terminal (74KKKKIKREIKI84), while in the regulatory subunit is located at the C-terminal (175RPKRP179).

2.4.2 STRUCTURAL FEATURES OF CK2

CK2 is a tetrameric holoenzyme composed by two catalytic subunits α and two regulatory subunits β. In vivo $\alpha_2\beta_2$ complex, $\alpha\alpha'\beta_2$ and $\alpha'_2\beta_2$ subunits exist either as isolated or as aggregated entities. From the structural point of view, the holoenzyme is similar to a butterfly.

2.4.3 STRUCTURE OF CATALYTIC SUBUNITS (α/α')

The complete amino acid sequence of the subunits α and α', deduced from cDNA of several species, has allowed us to identify all the 12 subdomains and residues of kinase domains that are highly conserved within the large family of protein kinases.
The comparison of the sequence of α subunit in distantly related organisms such as yeast, *Drosophila melanogaster* and human, has shown a remarkable degree of similarity of primary structure. In particular, the amino acid identity between yeast, *Drosophila melanogaster* and human is the 90% without considering the last 53 amino acids in the human sequence, absent in *Drosophila melanogaster*. The catalytic subunit of CK2 shows a great similarity with the catalytic domain of protein kinases cyclin-dependent (CDKs), with Mitogen Activated Protein (MAP) kinase and Glycogen Synthase Kinase-3 (GSK-3), being in the group called CMCG[10]. A common characteristic shared by all members of this group, is represented by the presence of two inserts, a smaller one between subdomains IX and X and a wider between subdomains X and XI. The detailed description of the different regions of the catalytic subunit, was performed, for the first time, with the definition of the structure of the CK2α subunit of *Zhea mays*, reproduced in figure 4[70]

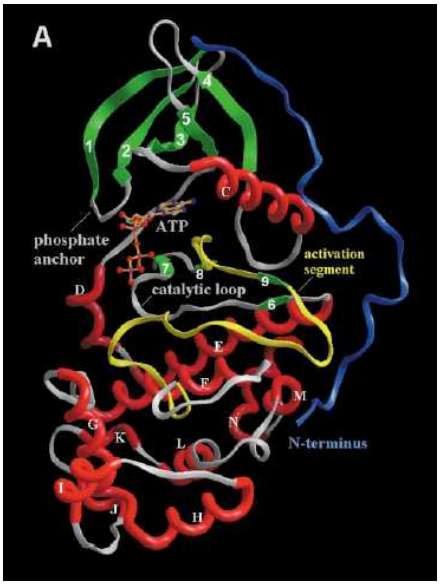


Figure 4: Global view of recombinant maize CK2α (rmCK2α). To make the contact visible between the N-terminal region (blue) and activation segment (yellow). The position of the active site is marked by the bound ATP molecule. The α-helices are in red and the β-sheets are in green.

The global structure of rmCK2α is a variant of the common bilobal architecture of protein kinases, with a β-rich N-terminal domain, an α-helical C-terminal domain, and the active site in the cleft between the two lobes. The N-terminal lobe ends at Asn117 and comprises the β-strands 1–5 and helix α C, while the rest of the molecule (the bigger portion) belongs to the C-terminal lobe.

A unique feature of CK2 is represented by the N-terminal region, stretching between residues Glu36 and Ser7, that seems to play a key role in stabilizing the conformation, joining the two lobes and forming well-defined contacts mainly with the activation segment.

Considering the amino acid sequence of human CK2α subunit and following the nomenclature already established, on the basis of several published studies on the structure of protein kinases, some groups of amino acids are particularly significant as noted in figure 5:

- **N-terminal segment**: it is a typical feature of CK2 because, unlike that of other protein kinases, it makes molecular contacts with the α-helix C and the activation loop maintaining the open conformation. The α-helix C in CK2 is a

structural element of particular importance because, as mentioned in the previous paragraphs, it is involved both in recognition of the substrate and in the binding of the β subunit. With a function similar to that of cyclin in CDKs, the N-terminal segment of human α subunit stabilizes the activation loop in the open conformation[71]. This activating effect is lost when the α subunit interacts with β. It is reasonable therefore the hypothesis that the “stabilizing" interactions, performed by the N-terminal segment of the α subunit, are replaced by other equally "positive" interactions with the β subunit, as soon as the heterotetramer is formed. This result is supported by biochemical evidence resulting from experiments with mutants of α and β subunits[71]. These studies also suggest that in the N-terminal segment (possibly at Ser28) could be located the site of "self-phosphorylation" showed by the α subunit upon incubation with polycationic compounds[72];

- **glycine rich loop**: as observed in all other kinases (and therefore also in the maize CK2α), this segment makes contacts with the β phosphate of linked ATP. The glycine rich loop is located in subdomain I and it is defined as "phosphate anchor". This segment is slightly different from the GXGXXG motif, common in many other kinases, because only the first two glycines of the motif are preserved, while the third glycine is replaced by a hydrophilic residue of serine (Ser51). Other important residues are Lys49, which contributes to the recognition of some acidic chains located at position n+2 in the peptide substrate[73] and Tyr50, homologous to the regulatory Tyr15 of CDKs, the phosphorylation of which in CDK (besides that of the Thr14) suppresses the catalytic activity[9]. At the beginning of this chapter it has been said that CK2 is able to use as a phosphate donor both ATP and GTP[74]. In the structure of the maize CK2α, the active site is occupied by one molecule of ATP, the purine base of which is not involved in the formation of hydrogen bonds. Moreover the space available for the purine is rather small because of the steric occupancy due to the side chain of Ile66 located in subdomain II. It was also noted that the ribose moiety is rather flexible because it cannot form hydrogen bonds at hydroxyl groups in 2' and 3' due to the particular orientation of α-helix that, in maize CK2, is completely different from PKA, CDK2 or CK1.

```
                                                          I
MSGPVPSRARVYTDVNTHRPREYWDYESHWEWGNQKDDYQLVRKLGRGKY          50
****************************************      *****
N-terminal segment                                .  glycin-

                 II                III                IV
SEVFEAINITNNEKVVVKILKPVKKKKIKREIKILENLRGGPNIITLADI          100
***                         *************
loop                        substrate binding, NLS
                            heparin binding
                            interaction with the negative domain of β

                    V                        VIa
VKDPVSRTPALVFEHVNNTDFKQLYQTLTDYDIRFYMYEILKALDYCHSM          150

          VIb              VII
GIMHRDVKPHNVMIDHEHRKLRLIDWGLAEFYHPGQEYNVRVASRYFKGP          200
   ************                 ********  *******
    catalytic loop                activation      loop p+1
                                  loop
VIII          IX                          X
ELLVDYQMYDYSLDMWSLGCMLZVSMIFRKEPFFHGHDNYDQLVRIAKVLG          250
                                    ******
                                  small insert
TEDLYDYIDKYNIELDPRFNDILGRHSRKRWERFVHSENQHLVSPEALDF          300
                 ******************************
                            big insert
      XI
LDKLLRYDHQSRLTAREAMEHPYFYTWKDQARMGSSSMPGGSTPVSSAN          350

MMSGISSVPTPSPLGPLAGSPVIAAANPLGMPVPAAAGAQQ                  391
```

Figure 5: amino acidic sequence of α subunit of CK2

These observations could at least in part explain the apparent ability of CK2 to use indifferently ATP and GTP as phosphate donors[65];

- **basic segment 74-80 (α-helix C)**: between subdomains II and III there is a basic sequence (Lys74-Arg80), located upstream of a conserved glutamic acid which defines the subdomain III and downstream of a series of required gaps according to the alignment of Hanks and Quinn[75]. This so high concentration of consecutive basic residues is almost unique to CK2. Several experiments have shown that the sequence Lys74-Lys77 is implicated in the inhibition of CK2 by heparin but, most importantly, the sequence Lys79-Lys83 was demonstrated to be crucially involved in the recognition of the protein substrate especially with a crucial determinant located at position n+3[76,77,78,79,80]. The 74-83 basic cluster of CK2 is located in a domain corresponding to the PSTAIRE sequence of CDKs, at the beginning dell'α C-helix and implicated in the binding of cyclin A[81]. In analogy with what observed between CDK and cyclin A, the basic region 74-83 of CK2 might be involved in interacting with the regulatory β subunit. It was found[80] that the basic sequence 74-83 (as well as the sequence 191-198) interacts with an N-terminal acidic domain of the CK2 β subunit. However, as shown by 3D structure of CK2 holoenzyme discussed below, this interaction cannot occur within a single molecule of CK2 but seems to require a multimolecular organization of the kinase;

- **catalytic loop**: this short segment of CK2α is very similar to the corresponding segment of PKA and CDKs; this is also confirmed in maize CK2α. This region of subdomain VIb contains the conserved residue Arg155, which precedes Asp156 and it enables CK2α to be classified among "RD kinases". These kinases are usually activated by a phosphorylation event requiring some ionic interactions (charge neutralization) between arginine and negatively charged groups[82]. It must be underlined, however, that not all "RD kinases" are activated by phosphorylation: the neutralization of the charge of arginine can be actually achieved in different ways as exemplified in CK1 and in phosphorylase kinase[83,84,85]. In the case of CK2α the mechanism is not yet clearly understood. The subdomain VIb also includes another important amino acid, which, in most Ser/Thr protein kinase, is a residue of aspartic or glutamic acid (Glu170 in PKA). This residue is involved in the interaction with the position n-2 of the

substrate, usually occupied by basic residues. In CK2α, which is acidophilic in nature, it is replaced by a histidine residue (His160) although the recognition of the position n-2 seems to be not so important in the substrates of CK2. This histidine belongs to a series of four equally spaced histidines, a feature almost unique in CK2. In fact, although these residues are present in a highly conserved region, no other protein kinases show more than two of these histidines, and the significance of such distribution remains still obscure;

- **activation loop and p+1 loop**: the region between the triplets "DFG" and "APE" that define the subdomains VII and VIII in most protein kinases, both altered in CK2 in DWG and GPE respectively, consists of 2 major domains called "activation loop" (or "T-loop"), due to a threonine residue that is constitutively phosphorylated in active PKA, and "loop p+1". In most protein kinases the "activation loop" presents residues, the phosphorylation of which (either autocatalytic or sponsored by another kinase), is related to an increase of enzyme activity: after phosphorylation, in fact, a conformational change leads to the correct orientation of the residues involved in the interaction with protein substrate and with the phosphate[82]. In CDKs it has been observed that the T-loop prevents the access to the catalytic site in the isolated catalytic subunit, while in the complex with cyclin A, it assumes a conformation allowing Thr160 to be phosphorylated, thus leading to a rapid activation of the kinase. In CK2α no serine or threonine in the "activation loop" is available, and it has never been observed phosphorylation in this region. These observations probably explains why the free catalytic subunit of CK2 (differently from the free catalytic subunit of CDKs) is spontaneously active. Close to the C-terminal of the T-loop there is the "loop p+1". In most Ser/Thr protein kinases, it contains a triplet of hydrophobic residues that are replaced in CK2α by basic residues (Arg191, Arg195 and Lys198) which, as it will be confirmed below, make molecular contact with acid residues at position n+1 of the substrate. Mutational studies[78] and molecular modeling suggested that the third residue (Lys198) should play the main role in this respect[80]. It should be noted also that the activation segment is in contact with the N-terminal region and, as mentioned before, this interaction stabilizes an open conformation thereby ensuring the activity of the enzyme, without requiring ligands and/or phosphorylating events;

- **"small" and "big" insert**: they form a distinctive feature of all members of the CMGC group of protein kinases and are located between subdomains IX and XI. The substitutions on the "big" insert do not seem to alter the catalytic activity and also the ability to bind to the β subunit during the formation of the holoenzyme[78]. On the contrary and in analogy with CDKs, the "small" insert could be involved in the recognition of peptide substrate, since mutations in this region lead to a significant increase of the K_m for the peptide substrate[86,87].

2.4.4 STRUCTURE OF REGULATORY SUBUNIT (β)

β subunit shows an overall dimeric structure. The monomer consists of a body and a tail. The body is composed by the N-terminal α-helix and by the Zn^{2+}-containing domain while the tail forms the C-terminal segment. The tail is not in contact with the body within its own monomer but is stabilized by hydrophobic interactions with the body of another monomer and, in the tetrameric form of the kinase, with one of the two catalytic subunits. Since CK2 is a constitutively active kinase the regulatory subunit does not control the on-off switching, rather it mediates the response to some polybasic effectors such as polyamines and other polybasic peptides.The β subunit of CK2 does not show any similarity with other known protein kinases, except for the “stellate” gene product in *Drosophila melanogaster*, which has 38% homology with the β subunit of *Drosophila*[88]. The primary structure, shown in figure 6, has an irregular distribution of basic residues in the C-terminal moiety of the molecule, while, in the N-terminal region, acidic residues predominate.

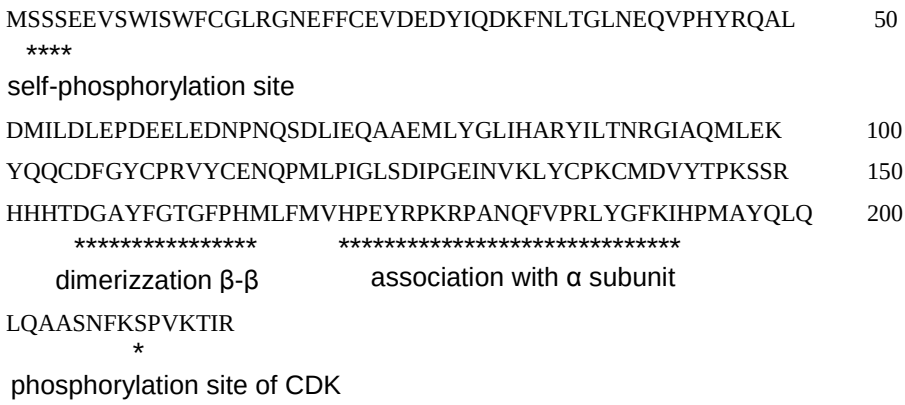


Figure 6: amino acidic sequence of β subunit of human CK2

Biochemical studies, strengthened by crystallographic evidence deduced from the 3D structure of a truncated form (1-180) of recombinant human CK2β[89] allowed to assign distinct functions to the N and C-terminal domains. A "zinc finger" domain containing four cysteines is implicated in the dimerization and a sequence very similar to the "destruction box" of cyclin B could modulate the protein stability. CK2 β is synthesized in excess with respect to the catalytic subunit and the formation of the β dimer is critical for the formation of the tetrameric complex. Furthermore, this subunit plays sometimes also a role as a modulator of the recruitment of substrates (p53, CD5, eIF2β) and regulators (FGF-2) of the α subunit. A critical function seems to be played, in particular, by the N-terminal region where some negatively charged residues seem to be involved in the negative regulation of CK2 (residues 55-64)[90,91]. This negative regulation is partially removed by interaction of CK2 with polybasic peptides (e.g. histones) and polyamines.

The β subunit has a self-phosphorylation site, MSSSEE, specific of this subunit, located at the very N-terminal end. While both the first and the second serine can be potential targets, only the first is apparently phosphorylated, indicating that the process of self-phosphorylation takes place mainly, if not exclusively, at Ser2[90,92]. The mechanism of this process was originally believed to be intramolecular, but recent evidence, mainly drawn from 3D structure analysis of the holoenzyme, demonstrated that it is instead due to the formation of higher order supramolecular structures. A number of biochemical studies provided evidence, on the other hand, that the C-terminal region is responsible for the positive regulatory activity, for the stabilization of the dimer β-β, for the association with the α subunit, and for the protection against denaturation and proteolysis[90,93].

The identification and characterization of these 2 domains of β subunits, summarized in figure 7, were performed by using different mutants of β subunit and a variety of synthetic fragments reproducing the C-terminal (155-215) and the N-terminal region (1-77). The same studies highlighted also a negative role played by the N-terminal tail especially with some substrates, for example with calmodulin, which can be phosphorylated by the holoenzyme only in the presence of a positively charged compound (e.g. polylysine) capable of shielding and removing this negative effect[94].

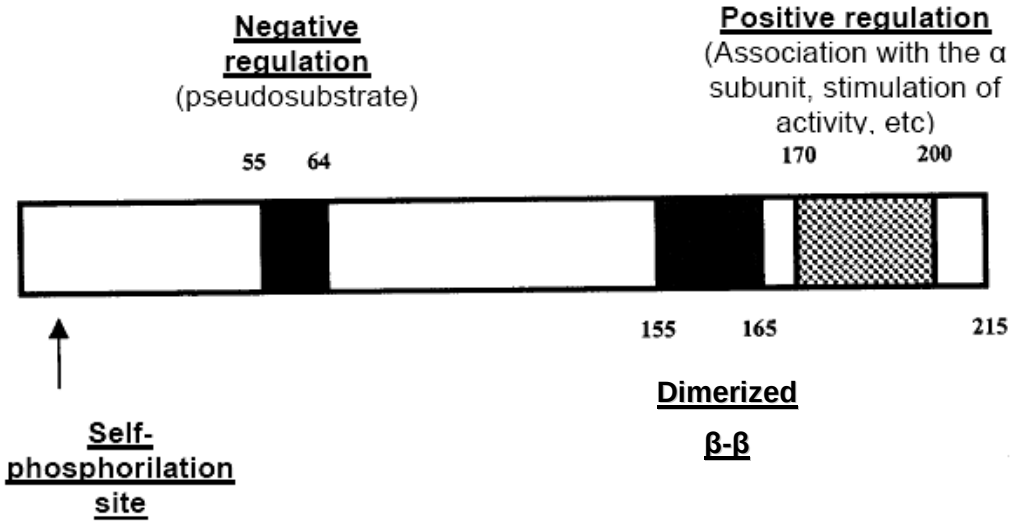


Figure 7: Schematic representation of functional domains of the β subunit of human CK2.

The discovery that C-terminal fragments of β subunit [155-215 peptide] (but not its shortened derivative 171-215) self-join and interact with the α subunit, as later confirmed by Far Western blotting experiments[94] identified the C-terminal part of the molecule as the crucial region to stabilize the association between the subunits in the heterotetrameric structure of CK2. It has to be finally remembered that at the end of the C-terminal domain a residue of serine (Ser209) is phosphorylated by CDKs in vivo and in vitro[95]; this serine is, however, absent in *Drosophila melanogaster*, yeast and *Arabidopsis* and its deletion is apparently without consequences[96].

The three-dimensional structure of β regulatory subunit has been resolved[89], confirming most of the biochemical evidences achieved during these latter studies. The molecule, which spontaneously crystallizes as a dimer stabilized by a “zinc-finger” (figure 8) is roughly composed by two domains in each of the two monomers:

- domain I (5-104 residues, N-terminal portion): it is totally composed by α helices (α1, α2, α3, α4 and α5). In this domain, rich of acid residues, it is possible to see:
  - an acidic pocket made by α1 and α3 helices that, within an acidic loop [55-64] is responsible of negative regulation of CK2 and of interaction with polycations.
  - the N-terminal region of α4 in which the self-phosphorylation site is present.

- domain II (105-161 residues, C-terminal portion): contains three antiparallel β strands and one α helix (α6). In this domain a motif, a “zinc finger”, appears to be implicated in the dimerization, due to thirteen hydrophobic side chains assuring non polar interactions between the two monomers.

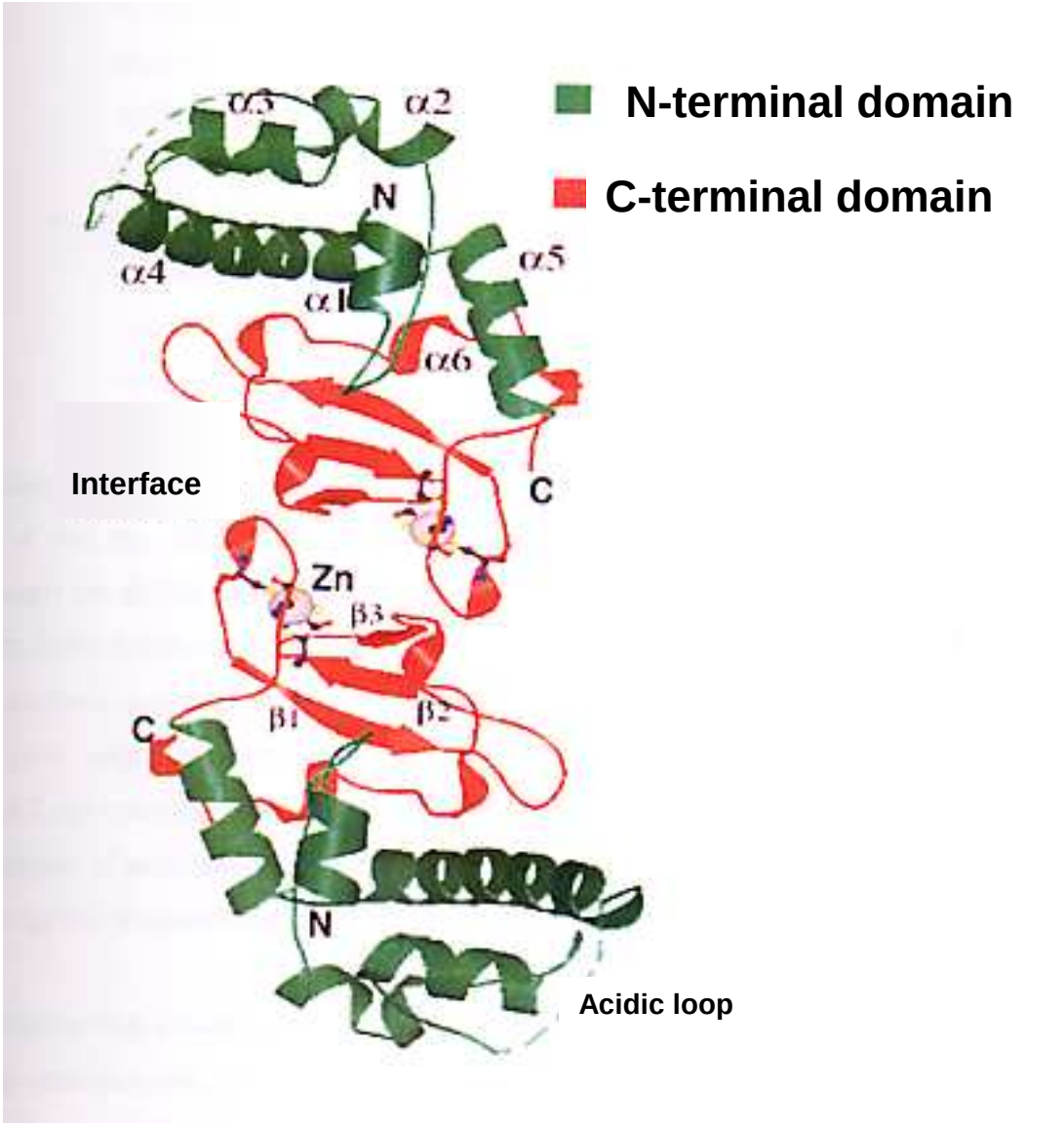


Figure 8: Three-dimensional structure of the dimer of human β subunit

2.4.5 QUATERNARY STRUCTURE OF CK2

As mentioned before, the protein kinase CK2 is a pleiotropic and ubiquitous enzyme found in all eukaryotic organisms so far examined. Contrary to most protein kinases, which are quiescent until they are activated in response to specific stimuli and effectors, protein kinase CK2 is constitutively active and independent from second messengers[17]. The enzyme has a heterotetrameric composition with a molecular weight of 130 kDa. As mentioned, the heterotetrameric structure is found in almost all forms of nuclear and cytoplasmic CK2 examined, but there are several exceptions. Preparations of CK2 from human spleen appears to show the only presence of the monomeric form of 44 kDa; a similar situation was observed in *Zhea mays* [97] and in *Dictyostelium discoideum*[98] where only the catalytic subunit was isolated. A characteristic of CK2 in *S. cerevisiae* is the absence of the typical β subunit of 26 kDa and the presence of two regulatory subunits, β and β', both larger than the animal β subunits (with molecular weight of 41 kDa and 32 kDa, respectively) and expressed by separate genes[99,62]. Similar situation was found also in *Arabidopsis thaliana*[100].
Data obtained from several laboratories, based on 2H technique experiments, have documented a strong tendency of β subunit to interact, besides with itself, with the catalytic subunit and with a number of other intracellular "partners". On the contrary, α subunit cannot interact with another α subunit[63,101,102]. These observations, also emerged in the 3D structure of the two isolated subunits, were fully confirmed by the definition of the crystal structure of the heterotetramer[103] illustrated in figure 9.

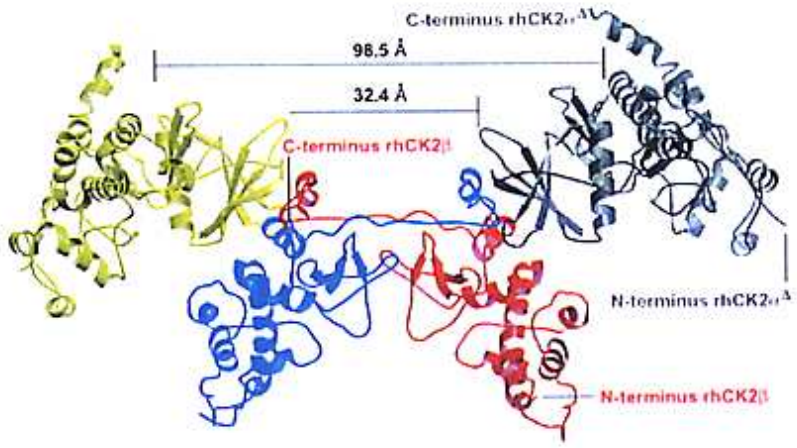


Figure 9: Representation of the three-dimensional structure of CK2 holoenzyme.

Of particular interest from the analysis of the crystals was the evidence that the individual β subunits do interact not only with each other to form a central “dimeric core”, but also, through their C-terminal ends, with both catalytic subunits. It is also important to remember that the formation of the heterotetramer leads to a stabilization of the enzyme since in general the activity of the human free α subunit is approximately 3-5 times lower than the activity of the holoenzyme. Of particular importance is also the observation that the N-terminal region of both the β subunits, which contains the site of self-phosphorylation, is too distant from the catalytic pocket of the two α subunits to explain an "intramolecular" mechanism. This observation was mainly responsible of further studies leading to the hypothesis of supramolecular re-arrangements.

2.4.6 SPECIFICITY AND CATALYTIC PROPERTIES OF CK2

As already mentioned, CK2 is one of the few protein kinases able to use also GTP as phosphate donor although the physiological implication of this property is still understood. The specificity towards GTP seems partially due to the presence of Val166 (Ile66 in α maize) and Ile174, that in other kinases are almost invariably replaced by Ala and Phe. The considerable bulkiness of Ile and Val as compared with Ala and Phe could also explain, on one hand, the insensitivity of CK2 to staurosporine, a very powerful competitive inhibitor for ATP of most protein kinases[91] and, on the other, the greater susceptibility to halogenated derivatives of benzimidazole and of benzotriazole[92,104,105].
Coming back to the term "specificity", it implies the ability of the kinase to choose the appropriate site in the appropriate substrate. This parameter can be evaluated considering 2 aspects:

• Substrate specificity: the substrate specificity of CK2 has been evaluated both by identifying along the years the features of the phosphorylated targets and by using synthetic peptide substrates. The first indication came from the identification of the subunits phosphorylated within casein, and subsequently it becames evident that CK2 prefers acidic proteins (e.g. casein and phosvitin), rather than histones and protamines as artificial substrates[106,107];

• Site specificity: it refers to a number of factors that determine the recognition by a protein kinase of only one or a few amino acids into the protein sequence, as a target of phosphorylation. It can be assumed that the structural requirements are in principle inherent to the primary structure, and, more in general, to the secondary and tertiary structure of the target protein.
By several studies conducted with synthetic peptides and by a more recent comparative analysis of all the phosphorylation sites identified in CK2 substrates[17] it is fully confirmed that this protein kinase greatly prefers acidic sequences rich in glutamic and aspartic acid.

It is interesting to note that the phosphorylated residues (especially serine and threonine) can replace carboxylic residues as specific determinants suggesting a critical role of electrostatic contacts between the kinase and the negatively charges located in close proximity of the phosphorylatable residue. This includes CK2 in a small group of Ser/Thr kinases “phosphate-directed", able to adopt a synergistic mechanism of phosphorylation implicating two protein kinases, where the first kinase creates the site for subsequent phosphorylation, performed by the same enzyme or by another kinase[22].
The minimal consensus sequence of CK2, identified by numerous biochemical studies with synthetic peptides[108,109] is below summarized:

$$\mathbf{X_{n-1}\text{-}S/T\text{-}X_{n+1}\text{-}X_{n+2}\text{-}E/D/Sp/Yp}$$

A number of fundamental principles can be underlined:
a) the preferred phosphoacceptor residue is the serine, followed by threonine and very rarely tyrosine;
b) the phosphorylation catalyzed by CK2 is usually specified by multiple acidic residues located downstream of the phosphorylatable amino acid;
c) the residue at position n+3 plays a key role;
d) of crucial importance is also the position n+1 where an acidic residue has been found in 75% of cases. Whenever the n+1 acidic determinant is missing, it is usually found at position n+3 and vice versa;
e) additional acidic residues, located between positions -4 and +7, behave generally as positive specificity determinants[110,111,112,113]. The efficiency of CK2-mediated phosphorylation is critically dependent on the extension of the acidic C-terminal

sequence; in fact each site of phosphorylation of CK2, found in natural proteins, is surrounded in average by 5.2 acidic residues;

f) structural effects ascribable to the tertiary structure of the protein substrate may sometimes “place” at position n+3 acidic residues otherwise distantly located;

g) as mentioned above, phosphate groups of phosphoaminoacids, especially of Ser-P and Tyr-P, can be recognized as specificity determinants[43] with the same efficiency of the carboxylic side chains, and the order of preference was found to be: Tyr-P>Ser-P>Asp>Glu>Thr-P;

h) among the potential “negative” determinants there are basic residues at any position close to the target Ser/Thr and the presence of a proline at position n+1. To note that a prolyl residue is conversely essential for the CDKs consensus sequence[115].

i) in a few cases CK2 was found to phosphorylate also tyrosyl residues. Especially relevant appeared the phosphorylation in vivo and in vitro documented in nuclear immunophilin Fpr3 of the yeast where the previous phosphorylation of Ser186 by CK2 provides the specificity determinant required for the subsequent phosphorylation by CK2 itself of Tyr184[114].

Table3: Summary of the characteristics of the CK2 natural phosphosites.

Total number of substrates:	**177**
Total number of sites:	**308**
Serine sites:	**266 (84%)**
Threonine sites:	**41 (13%)**
Tyrosine sites:	**1 (0.3%)**
Sites missing acidic determinant in n+3:	**43 (14%)**
Sites missing acidic determinant in n+1:	**88 (29%)**
Sites missing both acidic determinant:	**8 (2.5%)**
Average number of acidic residues for each site:	**5.2**
Sites with more than two acidic vicinal residues:	**270 (88%)**
Sites with only one acid residue:	**9 (at +3 position)**

2.5 REGULATION OF CK2

Since its early discovery a number of investigations were performed on CK2 with the aim of understanding its physiological role and the possible mechanism(s) of regulation. But the ubiquitary distribution and marked pleiotropy rendered this goal very problematic. Despite the structural similarity with PKA whose inactive heterotetramer is activated after dissociation promoted by cAMP, CK2 holoenzyme is constitutively active and can be dissociated only under drastic conditions. Moreover, no second messenger, no phosphorylation pathway, no clear regulator was known.
It is therefore reasonable to suggest the existence of different regulatory mechanisms according to cell types, subcellular distribution or the particular phase of cell cycle.
Nevertheless, at least three types of modulators of CK2 activity can be listed:

- **Modulators of activity of CK2 that interfere with recognition of protein substrate**. These compounds (some of which are shown in table 4) can be grouped as positive or negative effectors of CK2 activity. Activators are in general positively charged compounds including, among others, polyamines (spermine and spermidine), histones, protamine and other basic polypeptides characterized by high content in lysines and/or arginines. As already mentioned, all these compounds are more or less interacting with the acidic N-terminal domain of β subunit removing its down-regulatory effect. Polyamines are particularly interesting because of their known physiological role; it has been noticed, in this respect, that their biosynthesis parallels the stimulation and proliferation of cell growth[74]. The degree of stimulation is variable depending by the overall conformation of the effector as well as by the number of charges and with some substrates (for example with calmodulin) it can be “all-or-nothing”[116]. Inhibitors, on the other hand, are in general polyanionic compounds bearing a variable number of negative charges able to interfere with the binding of protein substrate by making electrostatic contacts with some basic residues located in the active site of the kinase and therefore competing with the acidic specificity determinants located around the substrate phosphoacceptor site.

ACTIVATORS	CONCENTRATION (AC_{50})
Polyamines (spermine)	280 μM
Basic polypeptides (polylysine)	0.4 μM
INHIBITORS	**CONCENTRATION (IC_{50})**
Heparin	0.5 μM
Ialuronic acid	0.7 μg/ml
Heparin sulphate	5.4 μg/ml
Dermatan sulphate	43 μg/ml
Polyglutamic acid	10 μM
Poly Glu/Tyr (4:1)	0.07 μM

Table 4: Effectors able to modulate in vitro the activity of CK2

Unlike the positive regulation by polycations, the inhibition made by polyanions can usually be observed both on isolated catalytic subunit and on the holoenzyme. Besides the well known sensitivity to heparin, the inhibition given by glutamic acid and aspartic acid random co-polymers, is reminiscent of the dual specificity of this kinase[117].

- **Modulators interfering with recognition of nucleotide substrate.** The search of "competitive inhibitors" of nucleotide phosphate donor, in these last years, has received great attention with all protein kinases. CK2 has been one of the most investigated in this respect.

  Numerous recent studies in fact, mostly performed in our laboratory, allowed to find a number of very effective and selective inhibitors. These are in general ATP-mimetic inhibitors, able to compete with phospho-donor substrate at the catalytic site of the α subunit of the enzyme. Their efficiency is given by the ability to interact with the specific residues located around of the binding site of ATP thus interfering with the correct normal interaction of the nucleotide.

Among the most relevant and interesting scaffolds proved to be effective inhibitors of CK2 there are:

- polyphenolic compounds (anthraquinones, flavonoids, hydroxyl-coumarins),
- polyhalogenated derivatives of benzimidazole and benzotriazole.

The starting point for the studies of the polyphenolic derivatives was emodin (1,3,8-trihydroxy-6-methyl-anthraquinone, figure 10), a drug (active principle) extract of *Rheum palmatum*, used in the East for the treatment of inflammation and for its anticancer properties.

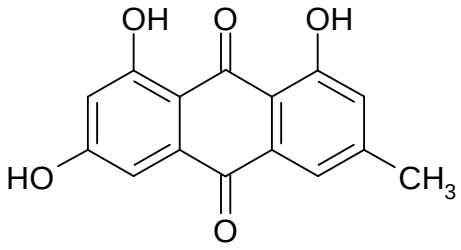


Figure 10: Chemical structure of Emodin

From the analysis of the interaction between αCK2 of *Zhea mais* and emodin, new inhibitors have been identified including, among others, MNA (1,8-dihydroxy-4-nitro-anthraquinone) and MNX (1,8-dihydroxy-4-nitro-xanthen-9-one) reported in figure 11 and 12[118].

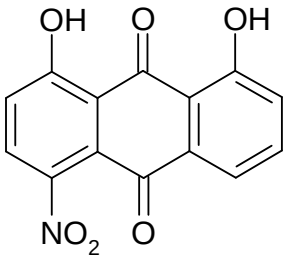


Figure 11: Chemical structure of MNA

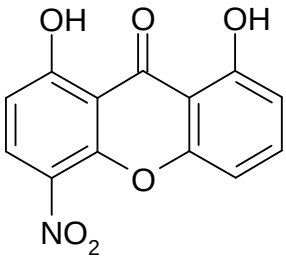


Figure 12: Chemical structure of MNX

The good efficacy of these compounds is related to the presence of a nitro-group which increases the dissociation constant value (Ka) of phenolic groups, favouring the interaction between the anionic form of hydroxyls and the kinase, in particular with Lys68 and Asp175[118].

By screening of a library of over 200 compounds other anthraquinones and other molecular scaffolds were later identified, among which DAA (1,4-diamino-5,8-

dihydroxy-anthraquinone) (figure 14) and DBC (3,8-dibromo-7-hydroxy-4-methyl-chromen-2-one) (figure 13) proved to be particularly active.

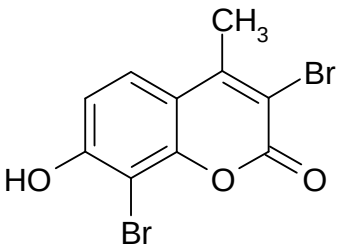


Figure 13: Chemical structure of DBC

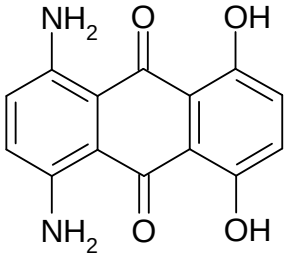


Figure 14: chemical structure od DAA

DAA interacts with the hinge region of the active site, establishing two hydrogen bonds with Glu114 and with Val116 that are usually responsible of the interactions with the adenine moiety of ATP[119].

By opening the cyclic ester of the coumarin a series of E/Z polybromurated cinnamic acids have been developed as well as, following a molecular simplification strategy, polybromurated benzoic acids and salicylic acids[120]. The most important candidate of this group is TBCA ((*E*)-2,3,4,5-tetrabromo-cinnamic acid).

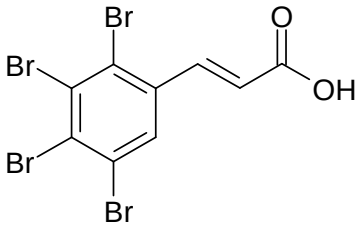


Figure 15: Chemical structure of TBCA

The interest for the halogenated derivatives of benzotriazole and benzimidazole is started in the 1990, when the inhibitory activity of DRB (dichloro-ribofuranosyl-benzimidazole) toward casein kinases has been demonstrated. However, under conditions usually adopted for CK2 inhibition, the compound resulted cytotoxic.

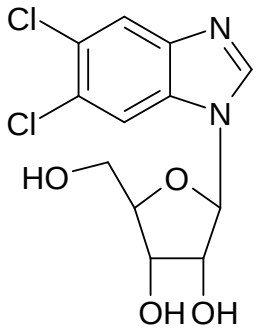


Figure 16: Chemical structure of DRB

With suitable structural modifications to increase potency and selectivity TBB (4,5,6,7-tetrabromo-*1H*-benzotriazole, figure 17) was firstly identified able to accommodate within the hydrophobic pocket partially overlapping to the binding site of ATP[121,122].

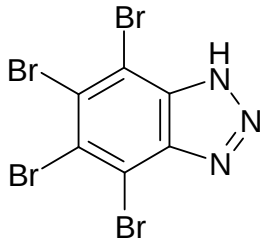
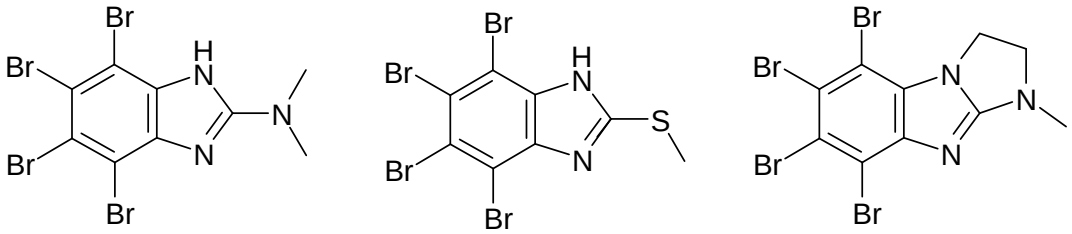


Figure 17: Chemical structure of TBB

Recently, the inhibitory power of TBB was significantly improved by the synthesis of a number of benzimidazole derivatives. Some of the most effective were K25 (dimethyl-(4,5,6,7-tetrabromo-*1H*-benzimidazole-2-yl)-amine) (DMAT), K37 (4,5,6,7-Tetrabromo-2-methylsulfanyl-*1H*-benzimidazole) and K44 (5,6,7,8-Tetrabromo-1-methyl-2,3-dihydro-*1H*-benzo[d]imidazo[1,2-a]imidazole)[123] (figure 18).



a **b** **c**

Figure 18: Chemical structure of K25 (a), K37 (b) and K44 (c)

These compounds all accommodate similar to TBB in the nucleotide binding site, but with deeper interactions hitting also Glu114 and Val116 of the hinge region. DMAT was among the first compounds chosen for cellular studies on CK2 inhibitors.

	Name	CK2 IC50 (μM)
Emodin	1,3,8-trihydroxy-6-methylanthraquinone	1.30
MNX	1,8-dihydroxy-4-nitro-xanthen-9-one	0.40
MNA	1,8-dihydroxy-4-nitro-anthraquinone	0.30
DBC	3,8-Dibromo-7-hydroxy-4-methyl-chromen-2-one	0.10
DAA	1,4-Diamino-5,8-dihydroxy-anthraquinone	0.30
TBCA	(E)-2,3,4,5-tetrabromo-cinnamic acid	0.11
DRB	dichloro-ribofuranosyl-benzoimidazole	23.0
TBB	4,5,6,7-tetrabromo-1H-benzotriazole	0.60
K25	dimethyl-(4,5,6,7-tetrabromo-1H-benzimidazol-2-yl)-amine	0.14
K37	4,5,6,7-Tetrabromo-2-methylsulfanyl-1H-benzimidazole	0.25
K44	5,6,7,8-Tetrabromo-1-methyl-2,3-dihydro-1H-benzo[d]imidazo[1,2-a]imidazole	0.74

Table 5: List of some selected inhibitors of CK2

Among natural polyphenolic compounds (e.g. derivatives of tannic acid, flavonoids and coumarins) a derivative of tannic acid, the ellagic acid (2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione) (shown in figure 19) appeared as one of the most potent and selective inhibitors of CK2 (K_i = 20 nM; IC_{50} = 0.04 μM)[124].

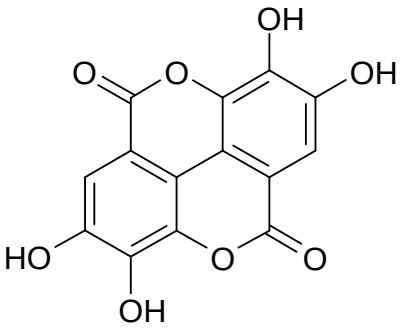
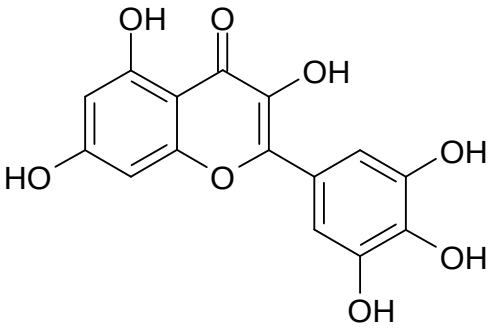


Figure 19: Chemical structure of ellagic acid

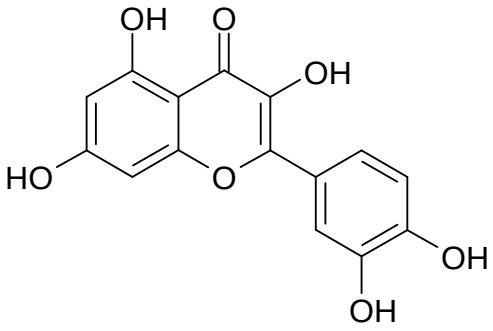
Many flavonoids behave as good inhibitors of CK2. They occur naturally in fruits, vegetables, cortexes, roots of many plants, but also in common green thea and wine[125]. They display in general some biological effects such as anti-allergic, anti-viral and anti-cancer activity as well as scavenging activity against free radicals[126].
Research in the field of flavonoids is increased since the discovery of the French paradox, i.e. the low cardiovascular mortality rate observed in Mediterranean populations in association with red wine consumption and a highly saturated fat intake[127].

Among the most important flavonoids proved to inhibit CK2 there are:

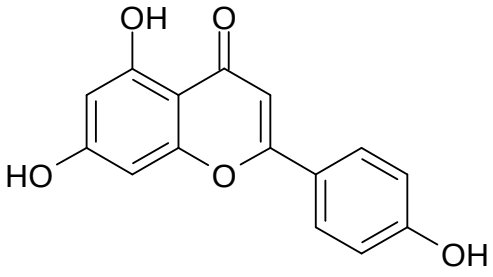
Myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxy-phenyl)-chromen-4-one)
(IC_{50} = 0.92 μM)



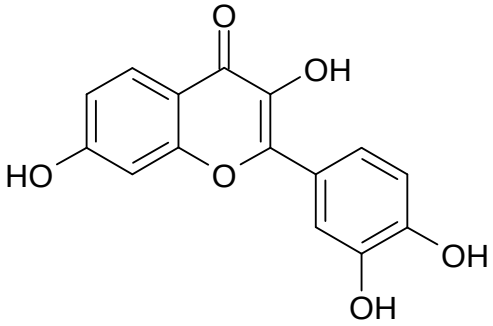
Quercetin (2-(3,4-dihydroxy-phenyl)-3,5,7-trihydroxy-chromen-4-one)
(IC_{50} = 0.55 µM)



Apigenin (5,7-dihydroxy-2-(4-hydroxy-phenyl)-chromen-4-one
(IC_{50} = 1.20 µM) (K_i = 0.74 µM)[128]



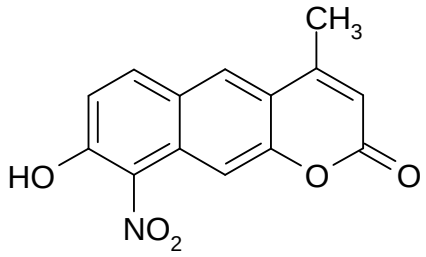
Fisetin (5,7-dihydroxy-2-(4-hydroxy-phenyl)-chromen-4-one
(IC_{50} = 0.35 µM)[129]



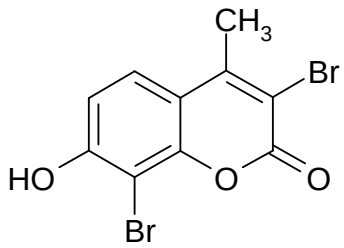
Like the flavonoids also coumarins are widely present in plants and were used as healing herbs since 980 BC[130]. They are present, for example, in plants such as Phytoalexine, in which they defend the plant from the microorganisms.
The hydroxyl derivatives of coumarins show anti-cancer activity[131].

A structure-activity study[118,132] highlighted the importance of the hydroxyl group at position 7 as well as the efficacy of the introduction of an electron attractor group at position 8 creating a negative charge necessary to bind the residue Lys168 of CK2. Similarly, a hydrophobic group at positions 3 and 4, such as a bromine or a methyl, proved to increase the inhibitor activity.
Among the most active compounds there are:

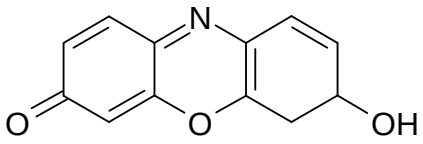
NBC (8-hydroxy-4-methyl-9-nitro-benzo[g]chromen-2-one)
(IC_{50} = 0.30 μM) (K_i = 0.22 μM)[118]



DBC (3,8-dibromo-7-hydroxy-4-methyl-chromen-2-one)
(IC_{50} = 0.10 μM) (K_i = 0.06 μM)[118]

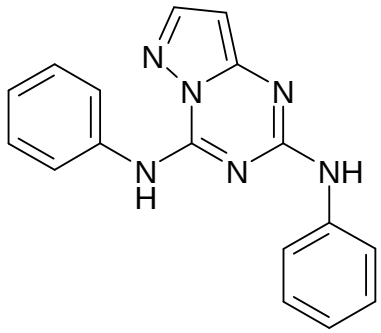


Also the structurally related **resorufin** (7-Hydroxy-6,7-dihydro-phenoxazin-3-one) was recently found to be a potent inhibitor CK2. Out of 52 kinases tested, only CK2 was inhibited in contrast to emodin, a structurally related, known CK2 inhibitor that, in addition to CK2, inhibited ten other kinases by 90%[133].
It shows an IC_{50} = 0.10 µM and a K_i = 0.06 µM

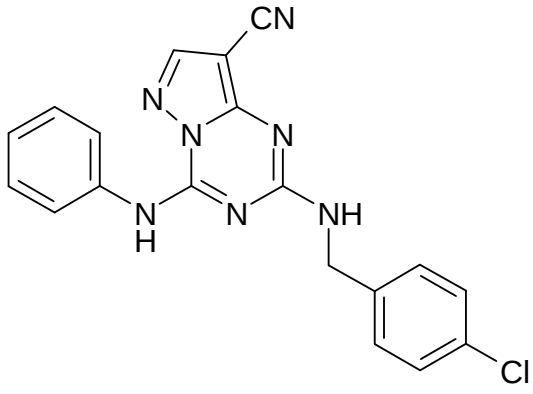


Among the most effective CK2 inhibitors ever identified there is the class of pyrazole-triazines. The more effectives, with inhibition values in the nanomolar range, are:

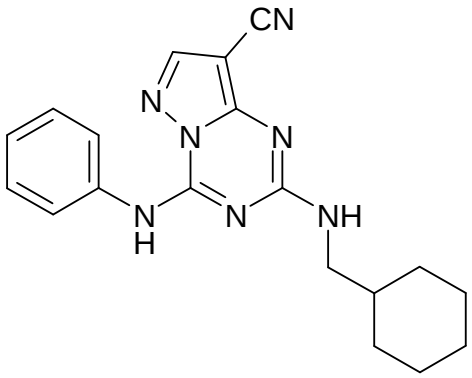
N*2*,N*4*-diphenyl-pyrazolo[1,5-a][1,3,5]triazine-2,4-diamine
(K_i = 0.26 µM)[134]



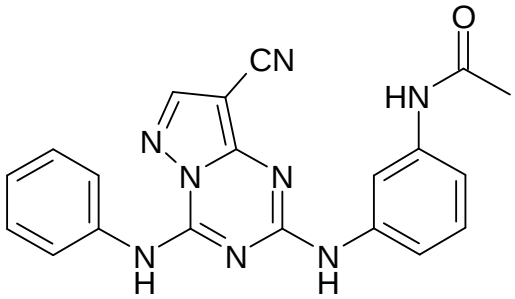
2-(4-Chloro-benzylamino)-4-phenylamino-pyrazolo[1,5-a][1,3,5]triazine-8-carbonitrile
(K_i = 0.005 µM)[134]



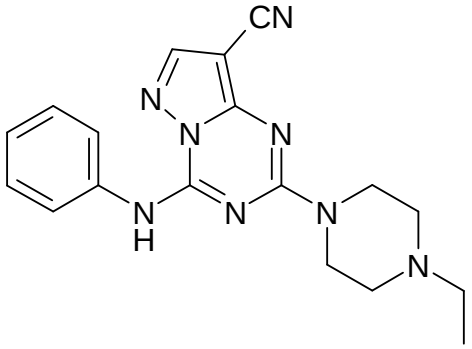
2-(Cyclohexylmethyl-amino)-4-phenylamino-pyrazolo[1,5-a][1,3,5]triazine-8-carbonitrile
(K_i = 0.0008 µM)[134]



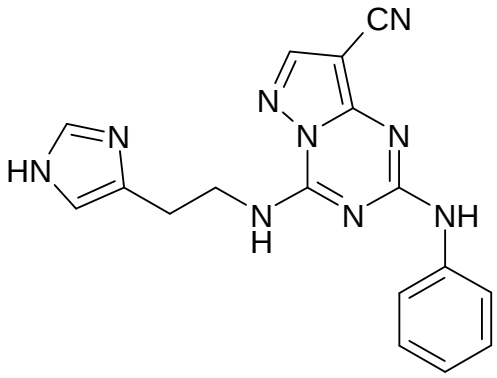
N-[3-(8-Cyano-4-phenylamino-pyrazolo[1,5-a][1,3,5]triazin-2-ylamino)-phenyl]-acetamide
(K_i = 0.0003 µM)[134]



2-(4-Ethyl-piperazin-1-yl)-4-phenylamino-pyrazolo[1,5-a][1,3,5]triazine-8-carbonitrile
(K_i = 0.24 µM)[134]



4-[2-(1H-Imidazol-4-yl)-ethylamino]-2-phenylamino-pyrazolo[1,5-a][1,3,5]triazine-8-carbonitrile
(K_i = 0.036 μM)[134]



The best inhibitor of this series is N-[3-(8-Cyano-4-phenylamino-pyrazolo[1,5-a][1,3,5]triazin-2-ylamino)-phenyl]-acetamide with a K_i = 0.35 nM, but in vivo it showed cytotoxic activity in prostate and colon cancers. The different activity observed between in vitro and in vivo experimentation is largely depending from the pharmacokinetic properties and in particular from the low permeability of the compounds.
Derivatives similar to pyrazolo[1,5-a][1,3,5]triazine as N & N1, have demonstrated to be a very potent, ATP competitive inhibitors.

- ❒ **“Allosteric” modulators of CK2**. Trying to understand the mechanism of regulation of CK2, a particular attention has been dedicated to the “cellular partners”, which are proteins able to interact either with the holoenzyme or with the free subunits, without necessarily undergoing subsequent phosphorylation by the kinase.

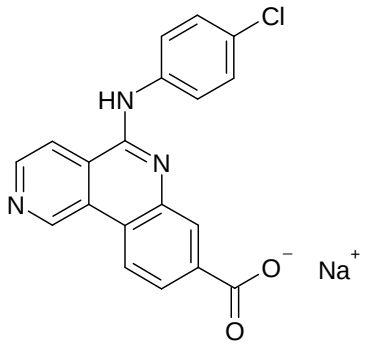
  This last event obviously implies the existence in the cell of free catalytic and/or regulatory subunits not assembled in the heterotetramer. The eccess of β with respect to α has been evoked to explain the interaction with other proteins[68], also because free β subunit is much more unstable and susceptible to the degradation. In the last few years the identification of new partners continuously increased after studies performed by using a a variety of approaches[65]. Among these there are nucleolin which has been one of the first identified natural partners, DNA topoisomerase I and II, ribosomal proteins such as L5 and L41

and proteins involved in the cell development such as ATF1, Dsg and ANTP. Of particular interest was found the interaction of CK2 with modulators of other kinases. For example it has been observed that c-Abl, able to phosphorylate and inhibit cdc-2 behaves similarly also with CK2α[135]. Moreover p21 (a potent inhibitor of CDKs) is also able to bind CK2β, blocking the activity of CK2[136]. In both latter cases the inhibition is obviously not due to a competition with the nucleotide phosphate donor opening a new field of investigation on the possibility that proteins able to specifically interact with the free subunits of CK2 could be in principle able to promote the dissociation of CK2 holoenzyme causing a variation of the activity provided that their affinity were comparable or even higher than that operating between the α and β subunits within the CK2 holoenzyme[137].

Clinical validation of CK2 inhibitors

In January 2009 Cylene Pharmaceuticals started the phase I of the clinical trials of the compound CX-4945 in patients with a “solid” advanced tumor or with multiple myeloma. This event represents the first and unique example of a CK2 inhibitor validated in clinical trials. CX-4945 is a derivative of 5-phenylaminobenzo[c][2,6]naphtyridin-8-carboxylic acid. It has been tested on more than 145 kinases displaying a remarkable selectivity for CK2 (IC_{50} = 2 nM).

In pre-clinical studies this inhibitor was found to promote tumor regression through a number of biochemical effects including block of cell cycle, activation of caspases and anti-angiogenic activity.



2.5.1 SUPRAMOLECULAR AGGREGATION AND ACTIVITY OF CK2

It has been underlined that protein kinase CK2 is a pleiotropic, ubiquitous and constitutively active kinase, despite of its quaternary structure generally target of strict regulation. However, sporadic observations made during late eighties, recently strongly supported by the inspection of 3D structure of CK2 holoenzyme, demonstrated that the enzyme aggregate at low salt concentrations giving rise to a filamentous structure of the kinase[138,139,140]. Self-polymerization is a reproducible and fully reversible process, which depends on the ionic strength of the medium and is closely relying on electrostatic interactions.

Sedimentation velocity analyses and electron microscopy[141], demonstrated the existence of four different oligomeric forms in aqueous solution. At high salt concentrations (0.5 M NaCl), a condition usually adopted during the purification of the kinase, CK2 appears as a sphere with an average diameter of 18.7±1.6 nm, corresponding to protomers $\alpha_2\beta_2$. At lower ion concentrations (0.2 M NaCl), protomers associate assuming ring-shaped structures probably composed of 4 protomers $\alpha_2\beta_2$. At 0.1 M NaCl, or even better in the absence of NaCl, CK2 is organized in thin and thick filaments. This response of CK2 to salt concentration is summarized in figure 20.

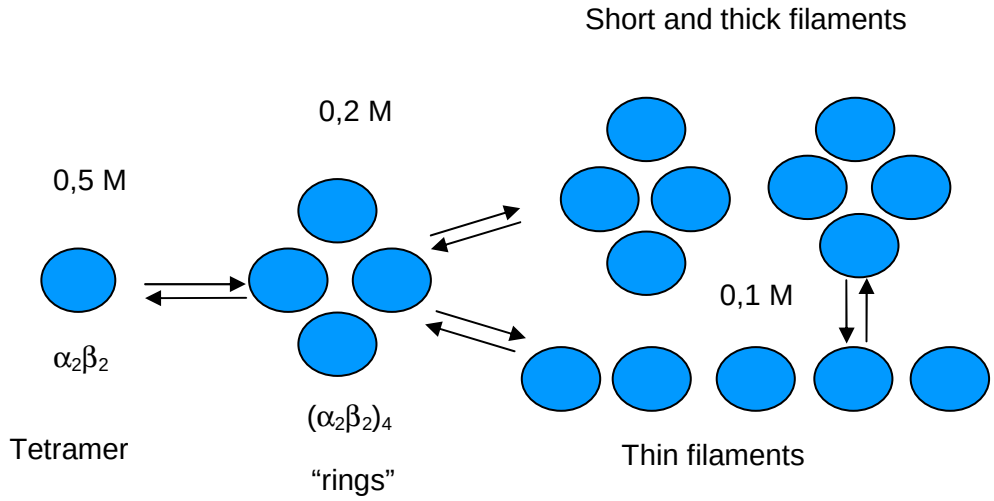


Figure 20: Forms of polymeric CK2 observed in vitro.

The tendency to molecular aggregation under condition of physiological ionic strength, together with structural considerations prompted several groups to better investigate the structure-activity relationship of CK2. These very recent studies demonstrated that:

1. maximal activity toward both artificial and endogenous substrates can be observed under physiological conditions, reproduced by a salt concentration of about 0.1 M, conditions favouring molecular aggregation of the kinase;

2. multimolecular aggregation is favoured by a "complementary" juxtaposition of two CK2 holoenzymes. Within this dimeric structure and even more within multimers of CK2 holoenzymes the heterotetramers are fitting into each other in such a way that the β subunit of a molecule is in molecular contact with the α subunit of an adjacent molecule.

3. the same interactions lead to a structural interaction between the site of self-phosphorylation of β subunit (Ser2) and the catalytic pocket of the α subunit of an adjacent molecule allowing the phosphotransferase process to occur. The self-phosphorylation, from this point of view, appears to be a test of molecular aggregation since it was never observed in the presence of isolated protomers which, however, under identical conditions still behave as “active” holoenzymes toward exogenous peptide substrates.

2.6 BIOLOGICAL ROLE OF CK2

The growing list of phosphorylable substrates identified in vivo and in vitro[17,18,21] together with ubiquitous distribution and pleiotropy corroborated the hypothesis that CK2 is essential for the survival of the cell[142]. A number of observations are in agreement with such a scenario. The importance of CK2 can be therefore examined under this point of view and its crucial role can be evaluated in the following general conditions:

- **SURVIVAL OF CELL**: in *Saccharomyces cerevisiae* the interruption of genes encoding for the CK2 catalytic subunit is lethal[144]. Similarly it was observed that in male mice CK2α' is produced mainly in the final stages of spermatogenesis and its block increases the number of apoptotic cells in testes, causing the infertility of the animals[145]. It has also seen that the deletion of both alleles for CK2β leads to deleterious effects in early phases of mouse development, hence the crucial role of CK2 in embryonic development and in the organogenesis. So the production of both subunits (catalytic and regulatory) of CK2 is essential for cell survival and CK2 has been proposed as belonging to a "squad survival"[23,142,146]. It is clear at this point, the potential relationship between CK2 and anti-apoptotic activity and, consequently, between high activity of CK2 and "abnormal" cell proliferation.

- **CK2 AND CANCER:** tumor development is linked to a deregulation of apoptotic and proliferative activity of the cell[147]. The relationship between CK2 and cancer is evident as several oncogenes, tumor and pro-apoptotic proteins are substrates of this kinase and accordingly high CK2 activity has been detected in tissues with neoplastic transformation.

Evidences in favour of the anti-apoptotic function of CK2 can be summarized as follows:

- phosphorylation of FAF1 (Factor associated to Fas). An important physiological mediator of apoptosis is Fas (membrane receptor of the family of TNF receptor). Under conditions promoting cell death, e.g. during DNA damage, CK2 appears to be a component of a molecular complex containing also FAF1 which is phosphorylated by CK2 and causes the block the expression of proapoptotic factors[148].

- Role of CK2 in Wnt signalling: Wnt, through a transmembrane receptor, inhibits the degradation of β-catenin; CK2 takes part in this system of intracellular communication, participating in a multi-protein complex with β-catenin which is

phosphorylated by CK2 and then transferred to the nucleus where it contributes to neoplastic development[149,150].

- CK2 can be an antagonist of the action of caspases (enzymes involved in protein degradation accompanying apoptotic processes). At least five proteins (Bid, Max, Connexin 45.6, HS1 and Presenilin-2), once phophorylated by CK2, become resistant to the attack of caspases.

- the evolution of neoplastic disease is directly proportional to the activity of CK2, a situation typically found in tumors from completely different organisms[21]. For example, elevated CK2 activity was detected in human leukemia cells[151] in murine lymphocytes transformed by virus[152] and in bovine cells transformed by the protozoan *Theileria parva*, which leads to diseases similar to leukemia[153].

In conclusion an altered, usually increased, CK2 activity is favouring the trend toward the transformation and uncontrolled proliferation. This alteration is, however, not dramatic since only slight changes have been observed. As a consequence, CK2 could therefore become a potential target for the detection of anti-cancer drugs, with the objective of a partial inhibition of the kinase in order to reduce and, hopefully, to suppress its oncogenic potential[21,154,155].

PROTECTION AGAINST THE CELLULAR STRESS: CK2 is supposed to be also responsible of intervention to protect cell from several types of damages, with special reference to DNA damage[156]. For example, it was demonstrated that CK2 is necessary for an efficient transcription of genes encoding tRNA by RNA-polymerase III (Pol III). Since transcription factor III-B is the key-component in the process mediate by Pol III and CK2 binds to this factor in corrispondence of TATA-binging-protein (TBP), in normal cellular conditions CK2 holoenzyme is bound to TBP through β subunit and the kinase allows an optimal transcription activity. A DNA damage, instead, promotes the dissociation of α subunit of CK2 from TBP, and consequently the block of transcriptional activity of Pol III[143].

PROTEIN "UNFOLDING": the CK2-mediated phosphorylation at certain sites could act as a stabilizer of the “unfolding” protein helices. For example in calmodulin or HIV Rev protein it has been shown that phosphorylation by CK2 causes the

breaking of a protein helix. The connection between CK2 and the destructuring of protein helices is even more interesting considering that the kinase could operate in the control point where proteins, coming from a tidy structure toward a disordered structure, are very susceptible to damaging conditions[157]. The presence, among the CK2 substrates, of proteins involved in neurodegenerative diseases, such as α-synuclein, prion protein and Tau protein could be relevant in this respect. The pathological potential of these proteins is in fact correlated to their ability to form insoluble aggregates.

PROTEIN-PROTEIN ADHESION: the possibility that Ser/Thr phosphorylation of proteins could in some way be implicated in protein-protein interaction machinery in a way similar to that promoted by Tyr phosphorylation in signal transduction represents an appealing event[109]. In fact, motifs were also identified, for which recognition is based on phosphorylation of Ser/Thr[158]. The activity of a pleiotropic kinase like CK2 could play in this context a crucial role.

CK2, ultimately, cannot be compared to the classical protein kinases and its extremely pleiotropic character could explain why it is also constitutively active. In future, the already long list of known substrates phosphorylated by CK2 could be enlarged. Evidence on the overall constitutive role of the enzyme in the cell, where it plays a wide variety of functions, from the control of gene expression, to synthesis and degradation of protein, to the maintenance of cell survival will be soon achieved.
While, however, the majority of protein kinases are active and work in a hierarchical and "vertical" way, following cascades of signals which go from the membrane to the nucleus, CK2 operates sideways, such as a free lance, damaging many ways of the signal to several different levels. As supposed 12 years ago, and recently confirmed by experimental data[143], in the case of CK2 the control of the activity would take part in a opposite way with respect to other kinases: while for most kinases "regulation=more work" for CK2 regulation=minor activity.

Chapter 3

Objectives

The research of new effective and selective inhibitors for a specific protein kinase represents, as we have seen before, a valid instrument to study its involvement in a specific cellular context.
The characteristics of an optimal inhibitor of protein kinases are, besides the ability to be easily carried into the cell, a good selectivity and a sufficiently elevated activity allowing a somministration at low doses.

In our laboratory, in these last years, a fruitful collaboration has been established between different groups for the optimization of synthesis, biochemical characterization and structural analysis of compounds proved to be effective on protein kinases with special reference to “casein kinases” CK1 and CK2. The main objective of my work was, therefore, to identify new powerful and selective inhibitors for these protein kinases by better exploiting the new achievements recently obtained.

Of invaluable importance was, in this context, the support of two laboratories of synthesis, namely the laboratory of Professor G. Zagotto at the Pharmaceutical Sciences Department of Padua University, where I performed some work during the chemical synthesis of a few compounds discussed in this thesis, and the laboratory of Professor Z. Kazimierczuk of the Warsaw University. Moreover, a crucial contribution to our work came from the computational studies in parallel performed at the Molecular Modelling Section by Professor S. Moro and by Dr. Giorgio Cozza at the Pharmaceutical and Biological Chemistry Departments of Padua University, respectively. Today a fairly good number of inhibitors for protein kinase CK2 are available and we recently started to develop also some inhibitors for protein kinase CK1. In particular, at the beginning of my work, we focused our attention on two distinct molecular scaffolds which have provided a number of very effective inhibitors: the polycyclic planar structure of ellagic acid, on one hand, and the polyhalogenated benzimidazoles on the other. In both cases, a threshold of efficiency in the low micromolar range had been reached. Moreover, the long lasting expertise in the field of anthraquinone chemistry, achieved by the group of Prof. G. Zagotto, suggested that this scaffold can be still useful, if accompanied by a suitable computational analysis, to find out new interesting inhibitors.

So, to achieve our main objective, the following sub-objectives were planned :

- The synthesis of new derivatives of ellagic acid, aiming at a simplification of the scaffold followed by a biochemical and structural characterization of their interaction with CK2 nucleotide pocket.
- The study of a new class of polyhalogenated benzimidazoles obtained through the replacement of bromine with iodine as substituent in the benzene ring; all the new derivatives, especially those obtained with suitable substitutions at positions 1 and 2 of the imidazolic moiety, should be biochemically characterized by determining kinetic parameters (IC_{50} e K_i) and selectivity.
- The biochemical characterization with activity and selectivity assays of new inhibitors of both CK2 and CK1 identified through a virtual screening of available large databases. This will be performed on a in-house panel of selected kinases (e.g. DYRK1a, PIM1 and HIPK2) and on a grand-scale screening on >70 protein kinases in collaboration with the laboratory of Professor Sir P. Cohen of the University of Dundee (Scotland) where I was planning to spend part of my PhD course.
- The identification of new compounds possibly active on specific isoforms of CK1, a field still unexplored in the literature.

Chapter 4

Materials and methods

4.1 ABBREVIATIONS

abs	absolute
℃	degrees centigrade
$CDCl_3$	Chloroform deuterate
$(CD_3)_2CO$	Aceton deuterate
CD_3OD	Methanol deuterate
$(CD_3)_2SO$	Dimethylsulfoxide deuterate
d	doublet
δ	chemical shift
dd	double of doublet
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
h	hour
HRMS	High resolution mass spectra
Hz	Hertz
J	coupling costant
m	multiplet
Me	methyl
MHz	Megahertz
min.	minutes
mmol	millimol
mol	mol
MW	Molecular Weight
^{1}H NMR	Proton's Nuclear Magnetic Resonance
^{13}C NMR	Carbon's Nuclear Magnetic Resonance
PMSF	phenylmethylsulphonylchloride
ppm	parts per million
rt	room temperature
s	singlet
t	triplet
t-Bu	*tert*-Butyl
THF	tetrahydrofuran
TLC	Thin Layer Chromatography

4.2 MATERIALS AND METHODS

Nuclear magnetic resonance (NMR) spectra were recorded on a *Bruker Avance AMX 300* spectrometer; ^{1}H and ^{13}C NMR spectra were run using $CDCl_3$: 7.26 (^{1}H) and 77.0 (^{13}C) ppm, $(CD_3)_2SO$: 2.54 (^{1}H) o 40,45 (^{13}C) ppm, $(CD_3)_2CO$: 2.05 (^{1}H) o 29.84/206.26 (^{13}C) ppm, CD_3OD: 3.31 (^{1}H) o 49.00 (^{13}C) ppm as solvent. As internal standard a signal of a solvent not completely deuterared has been utilized[159]. Chemical shifts (δ) are expressed in parts per million (ppm) relative to tetramethylsilane, and spin multiplicities are indicated as an s (singlet), br s (broad singlet), d (doublet), dd (double doublet), t (triplet), and m (multiplet) and the values expressed in Hz.

High resolution mass spectra were obtained using a Mariner^{TM} API-TOF (Perseptive Biosystems Inc.- Framingham MA 01701 USA) based on electrospray-TOF (Time of Flight) technology.

As stationary phase for column chromatography Merck's *Silica Gel 60* (230-440 Mesh) has been used.

Analytical thin-layer chromatography (TLC) was carried out on precoated silica gel plates (Merck $60F_{254}$), and spots were visualized with a UV light at 254 nm.

Analytical HPLC was carried out on Varian HPLC system by using a HP LiChroCART 125-4 mm LiChrospher 100 Rp-18 (5µm), with a particle size of 5 µm. The mobile phase was as following A: Phosphoric acid (0.15%) and B: acetonitrile; the eluting gradient was: $90_A{:}10_B \rightarrow 10_A{:}90_B$ in 30 min and then $10_A{:}90_B$ for additional 5 min. The eluate was monitored at 254 nm.

Aldrich and Fluka's reagents have been used without further purifications.

Aldrich, Fluka and Carlo Erba's solvents have been used without further purifications.

Aldrich's deuterate solvents have been used without further purifications.

During the biological assays the following materials have been used:

- **Enzymes**: **CK1 native** (nCK1) and **CK2 native** (nCK2), purified from rat liver; PIM1 and HIPK2 were recombinant product kindly provided by Prof. Sir P. Cohen (Dundee)

- **Isoforms** of **CK1**: isoform α of *Zebrafish* (Danio rerio) has been performed by Dr. Victor Bustos (Santiago, Chile), isoform γ1 of rat has been offered kindly by Dr. Peter Roach (Indianapolis, USA) while isoform δ of *Zebrafish*, which has been expressed and purified by Dr. Andrea Venerando (VIMM di Padua) in collaboration with Professor. J.E. Allende (Santiago, Chile).

- **Synthetic peptide subtrates**: the peptide **RRRADDSDDDDD** (CK2-tide)*;* **RRKHAAIGDDDDAYSITA** (CK1-tide); **RKRRQTSMTD** (PIM tide) were used for testing the activity of CK2, CK1 and PIM1, respectively. All these three peptides were synthesized by Dr. O. Marin (CRIBI, Padova); commercially available MBP (**myelin basic protein)** was used as substrate HIPK2.

- **[γ^{33}-P] ATP**: commercially available purchased from Amersham;

4.3 PURIFICATION OF PROTEIN KINASES CK1 AND CK2 FROM RAT LIVER

4.3.1 PREPARATION OF CYTOSOL

A typical preparation starts from 10 fasting rats. The livers were cut and washed in a solution containing 0.25 M sucrose and 0.05 mM PMSF. Washing was repeated at least two times. Then the material was homogenized in the same solution by using a *Potter* homogenizer. The homogenate was centrifuged for 20 minutes at 700 × g to remove nuclei and intact cells. The sediment was discharged, while the supernatant was centrifuged again at 9000 × g for 20 minutes. The new pellet mainly consisting of the mitochondrial fraction was discharged and the supernatant was finally centrifuged at 105000 × g (27000 RPM, Beckman ultracentrifuge) for 60 minutes. The clear supernatant obtained was then used as source for CK1 and CK2 purification.
Cytosol was 80% saturated with solid $(NH_4)_2SO_4$ following a centrifugation at 9000 × g for 20 minutes. The pellet was then redissolved with Tris-HCl 50 mM pH 7,5 and dialysed for 20 hours against a buffer containing 50 mM Tris-HCl pH 7,5, 0.25 M NaCl, 0.05 mM PMSF and 0,01% Brij 35.

4.3.2 P-CELLULOSE CHROMATOGRAPHY

The dialyzed cytosol was loaded on a P-cellulose column (25 x 5 cm), previously equilibrated with the same dialysis buffer. The column was exhaustively washed to remove unbound proteins at the starting ionic strength.
Bound proteins were then eluted from the column by a discontinuous gradient at increasing ionic strength (equilibration buffer plus 0.3, 0.5, 0.7 and 1 M NaCl).
Fractions (4.5 ml each) were collected from the column and tested for casein kinase activity by using casein as phosphorylatable substrate: a main peak of activity in corrispondence of eluition with NaCl 0.7 M was detected which was further purified by Ultrogel AcA34 gel filtration.

In the case of CK1 and CK2 which, as discussed above, are able to recognize the phosphate as specificity determinant, it seems that P-cellulose is working not simply as a typical ionic exchange column, but mainly as an affinity chromatography.
In fact, at the starting ionic strength of 0.25 M NaCl most cytosolic proteins do not bind to the column. On the contrary, protein kinases CK1 and CK2 are strongly retained by the phosphate groups of the column since a very high ionic strength is required for their elution[160]

4.3.3 CHROMATOGRAPHY ON COLUMN OF ULTROGEL AcA 34

A gel filtration on Ultrogel AcA 34 column (90×2 cm) was necessary to separate CK1 from CK2. In order to avoid self-aggregation of kinases, especially that occurring in the case of CK2 (as discussed above) this step of chromatography was performed at rather high ionic strength: in fact the equilibration and elution buffer was 0.1 M Tris-HCl pH 7.5 containing 0.5 M NaCl, 0.05 mM PMSF and 0.01% Brij 35. Under these conditions, the peak of casein kinase activity eluted from P-cellulose can be resolved in two peaks: the first, eluted in correspondence of about 140 kDa, and is represented by CK2, the second, of about 38 kDa, corresponds to CK1.
More in detail, the peak of activity collected during the previous purification on P-cellulose was concentrated by ultrafiltration (Diaflò, membrane UM10) and loaded on the column of Ultrogel AcA 34. Fractions of 5 ml were then collected and tested for casein kinase activity by using specific peptide substrates for CK1 and CK2, respectively.
The two peaks of activity were then collected, separately concentrated by ultrafiltration, dialyzed against 20 mM Tris-HCl pH 7.5 containing 50% glycerol and stored at -20℃.

4.3.4 CASEIN KINASE ACTIVITY ASSAY

To test casein kinase activity a typical mixture was prepared (final volume of 25 µl) containing:

- 50 mM Tris-HCl buffer at pH 7,5
- 12 mM $MgCl_2$

- 0,1 M NaCl
- Protein or peptide substrate substrate at suitable concentration (e.g. for CK2 assays, 100 µM RRRADDSDDDDD specific peptide was used).
- 40 µM [γ^{33}-P]-ATP, usually with a specific radioactivity of 500-1000 cpm/pmol.

The reaction starts with the addition of the kinase and the samples are incubated at 37℃. After 10 min (a typical incubation time used for CK1 and CK2 assays) the reaction is blocked by putting the sample in ice.
Then the protocol differentiates according to the type of substrate, aiming at the separation of the radiolabeled phosphorylated substrate from the residual radioactive ATP.
In case of small peptide substrates the procedures change according to the chemical characteristics of these peptides:

<u>Acidic peptides containing at least three basic residues of lysine or arginine.</u>
For this kind of peptides, commonly used for CK1 and CK2 activity assays, filters of P-cellulose were routinely adopted[162]. At the pH of the washing solution (0.5% phosphoric acid), phosphate groups of P-cellulose are still partially dissociated, and their negative charges can bind the positively charged groups of lysine or arginine of the peptide. (It is important to remember that at the same pH the carboxylic groups of the peptide are almost fully protonated).
In detail, assays were stopped by putting in ice before spotting aliquots on phosphocellulose filters (2 x 2 cm each). Filters were washed in 75 mM phosphoric acid (5-10 mL each) four times and then once in methanol or aceton and dried before counting.

<u>Acidic peptides devoid of basic residues.</u>
With this kind of peptides P-cellulose filters cannot be used to separate phosphorylated peptide which is conversely bound to DEAE filters under different conditions[114].
In this case, at neutral pH, the negative charges of the peptide (a minimum of 7-8 acidic residues are required on the peptide to assure stable binding) stably interact with amino groups of the filters. None of the peptide substrates used to test activity of the kinases considered in the present work, however, required this procedure.

On the other hand, if the phosphorylatable substrate is a protein with suitable size to be retained in SDS-PAGE, radioactive ATP can be easily separated by a simple staining-destaining procedure of the polyacrylamide gel.
In these cases, the radiolabeled substrate can be directly autoradiographed in an Istant Imager apparatus (Canberra Packard).

4.3.5 DETERMINATION of KINETICS PARAMETERS

Kinetic constants, obtained during the experiments described in this thesis, have been usually determined with the method of Lineweaver-Burk. V_{max} and K_m values were deduced from double reciprocal plots.
Usually in a typical experiment the concentration of the first substrate (ATP or protein/peptide) is varied in a range from 0.1 to 10 times the K_m, while the second substrate is maintained at a costant concentration and saturating for the enzyme.
The determination of the inhibition constant K_i has been obtained analyzing, first of all, the effect of different fixed concentrations of each inhibitor on the kinetics of ATP. Additional analyses of the data, by using a replot of K_m/V_{max} values against [I], it is possible to calculate K_i as intercept on the negative axis of the abscissa. Alternatively, in the presence of typical competitive inhibition, the K_i values can be calculated according to the equation of Cheng-Prusoff:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_M}}$$

Besides the direct calculation of K_i values from IC_{50} determination, a useful application of the Cheng-Prusoff equation is given by the possibility to run inhibition experiments at increasing concentration of the inhibitor at very low concentration of ATP. Working, for example, at 1 μM ATP, a concentration still compatible with the reproducibility of experimental data, the IC_{50} obtained from these experiments roughly corresponds to the K_i.

Chapter 5

Experimental section

5.1 ELLAGIC ACID AND ITS DERIVATIVES

Two are the main objectives which in these years have inspired the of new inhibitors of protein kinase CK2 in our laboratory:

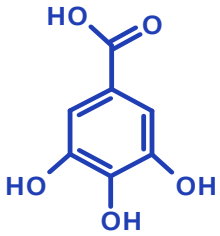
- the increase of the efficiency
- the selectivity

These parameters are usually considered to establish the quality of a new inhibitor.
Starting from 5,6-dichloro-ribofuranosyl-benzoimidazole (DRB), one of the first ATP-mimetic CK2 inhibitors[92], the research in this field was increased and in a few years the threshold of efficiency was lowered from the micromolar (DRB K_i = 23 μM) to the low micromolar (emodin K_i = 2 μM), until the nanomolar range with a new series of compounds among which one of the most effective appeared to be ellagic acid (K_i = 0.02 μM)[124].
Today a number of efficient inhibitors of this pleiotropic protein kinase are commercially available; most of them, however, do not display a remarkable selectivity with respect to other protein kinases known to be involved in important oncol-ogical and neurodegenerative diseases.
One of the main objectives of my work was therefore to search for more selective inhibitors of CK2, by screening a small panel of protein kinases available in our laboratory and by exploiting the expertise of P. Cohen's group during my stage in Dundee.

5.1.1 ELLAGIC ACID

At the beginning our attention was focused to the isolation of some derivatives of Gallic acid, a natural compound extracted from the plant ***Quercus infectoria*:**

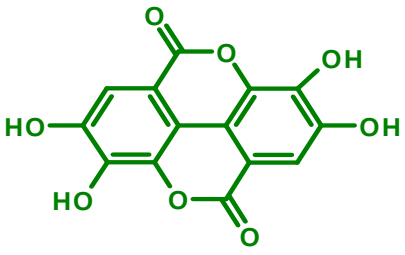
Quercus infectoria,



Gallic acid has an antioxidant property and it helps the cell to prevent oxidative damage. It shows to have a cytotoxic activity towards cancer cells[163] but also its astringent property in internal haemorrhages is known.
It is not considered, however, an inhibitor of CK2; in fact its IC_{50} is > 40 μM.

We started, therefore, to study gallic acid derivatives with the aim of finding out more efficient compounds. One of these was ellagic acid, a natural compound extracted from ***Punica granatum***; with a well known antioxidant property;

Punica granatum



Ellagic acid is a planar polyphenol present in nature as ellagitannins, which are molecules of ellagic acid esterified with glucose.
In several studies, ellagic acid has shown antineoplastic, antioxidant and antimutagenic activity. High quantity of this acid are present in strawberry, raspberry and in pomegranate, whose solid and liquid extracts are commercially available (powder, capsule and syrup) and used as extra diet.

Recently our research has discovered ellagic acid as a very potent inhibitor of CK2 (IC_{50} = 40nM)[124] so we planned the synthesis and biological characterization of derivatives of this interesting compound.
Since previous information obtained from the analysis of the crystal structures of CK2/inhibitor complexes suggested that optimal fitting of the inhibitor was assured by rigid and planar scaffolds, we planned to synthesize derivatives of Ellagic acid by maintaining its rigid structure.

From the structural point of view the ATP competitive inhibition of ellagic acid is due to the occupancy of a binding region between the N-terminal lobe and C-terminal lobe of the kinase, in the same way displayed by TBB (4,5,6,7-tetrabromo-*1H*-benzotriazole) and by other polyphenolic compounds. Figure 21 shows the 3D structure of ellagic acid in complex with CK2α catalytic subunit[164]:

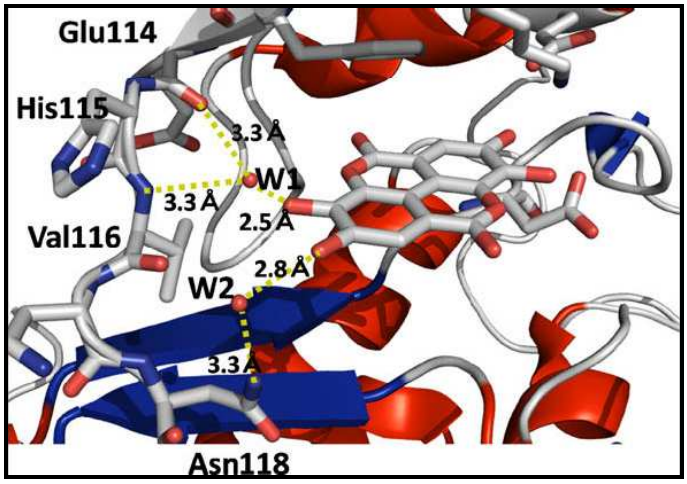


Figure 21: Interaction between ellagic acid and human CK2α at the hinge region. Ellagic acid binds to CK2α through the water molecules W1 and W2.

The molecule of ellagic acid shows an optimal steric and chemical complementarity with the enzymatic cavity. In the picture the interactions with the most important amino acid residues are highlighted.
Ellagic acid locates essentially on the plane found to be also occupied by other polyaromatic structures, but it is able to penetrate more in depth than other inhibitors to reach the hinge region of the kinase.
The most interesting structural feature of ellagic acid is therefore the ability to interact both with the hinge region and with some residues usually implicated in the binding of phosphate group of ATP at the same. This can be easily deduced by observing the 3D

crystallographic structure of the complex between human CK2 and ellagic acid, recently appeared in literature[164].

With this binding configuration, the hydroxyl at position 3 of Ellagic acid interacts with the carbonyl oxygen of Glu114 backbone through a water bridge. Similar contacts are occurring when the adenine moiety of ATP binds the active site of the CK2.

(shown in figure 22).

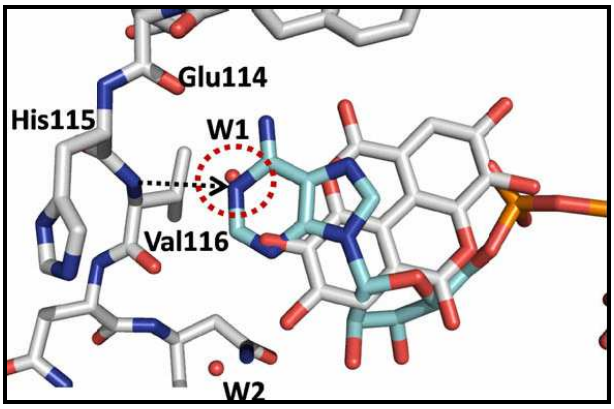


Figure 22. Superimposition of AMPPNP from 2PVR structure in PDB onto the ellagic acid–CK2α complex. N1 in the purine frame of AMPPNP fits the W1 water molecule.

The hydroxyl groups of ellagic acid at positions 7 and 8 interact with the carboxylic side chain of Asp 175, through an hydrogen bond.

Moreover, the hydrophobic interactions with Val53, Val66, Phe113, Met163 and Ile174 stabilize the ellagic acid-CK2 complex.

In conclusion both polar and apolar interactions of ellagic acid with the ATP binding site are responsible of its interesting inhibitory activity.

By the opening of one of the two lactonic rings of ellagic acid, we have obtained the monolactone derivative (figure 23); this derivative, however, proved to be a less powerful inhibitor of CK2 respect to ellagic acid. (IC_{50} = 0.2 μM)

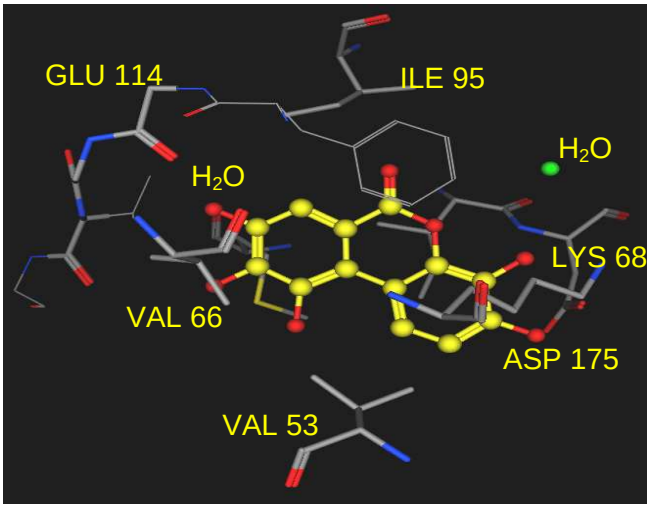


Figure 23: Docking of ellagic acid monolactone into the active site of CK2α.

The docking pose of the monolactone of ellagic Acid is quite similar to ellagic acid crystal structure: in fact the hydroxyls 3 and 4 interact with backbone carbonyl oxygen of Val66 and Glu114 through water bridges.

On the other side of the binding cleft the hydroxyl group at position 8 interacts with Asp175, while hydroxyl group at position 7, interacts with Lys68.

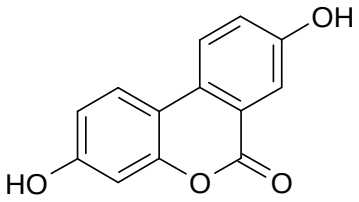
While hydrophobic interactions with Phe113, Met163 and Ile174 are still effective, no interaction with Val53 is now possible, and this could be the cause of the observed decrease of activity.

Altogether these latter findings suggested us to start with new strategies and, in particular, to take in consideration other derivatives of ellagic acid. This is the case, for example, of **Urolithin A**, known to be the bioactive metabolite of ellagic acid effect.

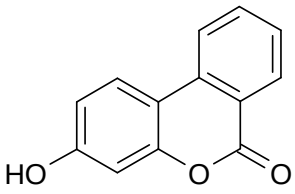
5.1.2 UROLITHINS

Urolithin is a natural dibenzopyranone extracted from the exudate of ***shilajit*** but, quite importantly, it is also a metabolite of ellagic acid.

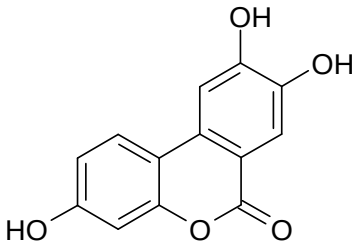
In literature four isoforms of Urolithin have been reported[165]:



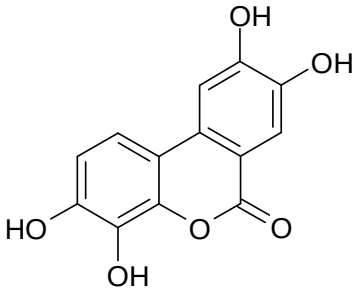
Urolithin A



Urolithin B



Urolithin C



Urolithin D

Shilajit, a panacea in oriental medicine, is an organic exudation from steep rocks (altitude 1200-5000 m) found in the Himalayas from arunachal Pradesh to Kashmir. It has also been found in other countries[166].

The organic matter of the *Shilajit* is formed by humus (consisting of hetero-polycondensates of plant and microbial origin metabolytes) mixed with their unchanged secondary metabolites.

Shilajit collecteded from Eastern, Central, and Western Himalayas was found to contain a number of common metabolites, among which the family of urolithin.

These compounds and some of their advanced intermediates were also encountered in *Euphorbia royleana* , and *Trifolium repens*.

It is also remarkable that urolithins were detected among the sediments of the organs of certain herbivores, and in sheep. According to Lederer[167] the special feeding diet of the beaver (its food mainly consists of buds and barks of trees) are responsible for these deposits.

5.1.2.1 PHARMACOLOGICAL ACTIONS

There are a lot of preclinical studies which confirm the numerous pharmacological activities of this natural compound[168]:

- antiulcer and antinflammatory: *Shilajit* seems to increase the ratio carbohydrates/proteins and decrease the number of gastric ulcer thanks to the increase of the mucus barrier and its efficacy in acute, subacute and cronic inflammations was demonstrated;
- antioxidant;
- promoter of learning: some studies on mice have demonstrated that this compound increases the learning and the memory;
- antidiabet activity: *Shilajit* is able to prevent the cascade of events leading to hyperglycemia;
- promoter of memory and ansiolytic: the subacute treatment with this compound in rats demonstrated a decreased turnover of 5-hydroxy-tryptamine and increased dopaminergic activity;
- antistress activity;
- antiallergic activity;
- immunomodulator activity;
- anti AIDS: patients under treatment have demonstrated an increase of CD4 and CD8 cells.

5.1.2.2 METABOLISM[165]:

Many health benefits of pomegranate products have been attributed to the potent antioxidant action of their tannin components, mainly punicalagins and ellagic acid. While moving through the intestines ellagitannins are metabolized by gut bacteria into urolithins that readily enter systemic circulation.

The contribution of human gut microbiota toward health improvement and genesis of various diseases has been widely recognized. One important function of intestinal bacteria is the fermentation of undigested food components leading to the production of metabolites of different physiological significance.

Human intestinal bacteria are able to metabolize dietary polyphenolic flavonoids by cleavage of the C-ring, hydroxylation, dehydroxylation, reduction of carbon-carbon

double bonds, and lengthening or shortening of aliphatic chains. Therefore, by the formation of different phenolic acids and other aromatic derivatives, gut microbiota can modify the bioactivity of the original compounds. In vitro fermentation of punicalagins and ellagic acid by human gut bacteria resulted in the formation of a dibenzopyranone urolithin A. Urolithin A and related analogs (figure 24) were also confirmed as intestinal microbial metabolites of dietary ellagitannins in animal studies.

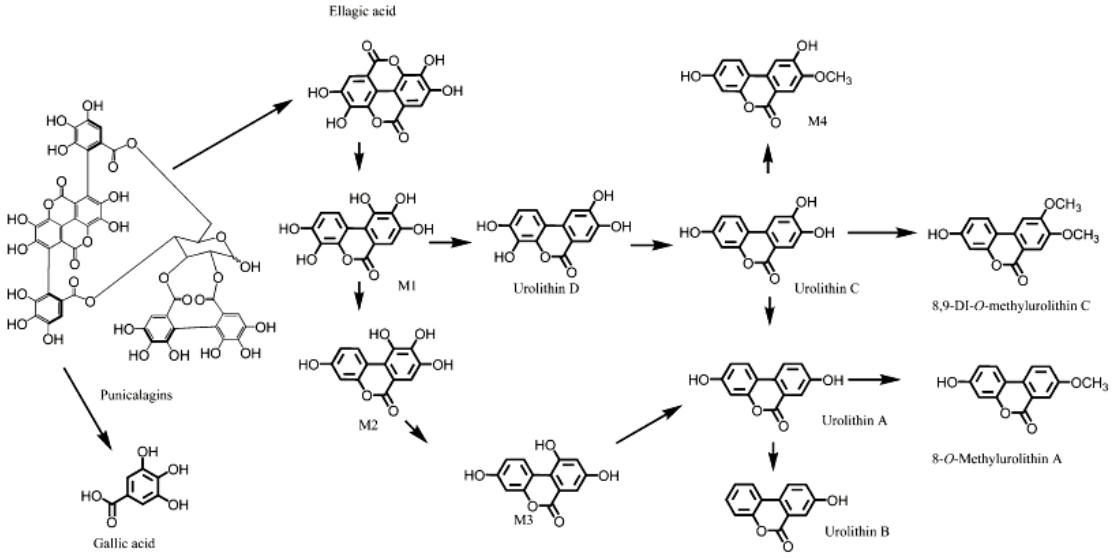


Figure 24: Proposed pathway of punicalagins transformation to urolithins by intestinal bacteria.

Common bacterial metabolites of various ellagitannins indicate that ellagic acid, the product of hydrolysis of ellagitannins, may serve as substrate in the formation of urolithins.

Urolithins appear in human systemic circulation within a few hours of consumption of pomegranate products, reaching maximum concentrations between 24 and 48 h. They are present in the plasma and urine for up to 72 h, in free and conjugated forms. Therefore, microbial metabolites could account for the increased antioxidant properties of plasma in human volunteers after consumption of pomegranate products.

Studies on the antioxidant activity have underlined that both urolithins C and D had higher antioxidant potency than ellagic acid and the punicalagins with IC_{50} values of 1.1 and 1.4 μM, respectively. They also showed higher activity than vitamin C. The dihydroxydibenzopyranone, urolithin A, with hydroxy groups at C-3 and C-8, exhibited less significant antioxidant activity (IC_{50} = 13.6 μM), while the monohydroxylated urolithin B did not show antioxidant activity.

In figure 24 there is the proposal pathway to account for the formation of the urolithins in the intestinal tract. The ingestion of ellagitannins involves abiotic hydrolysis at the pH levels of the small intestines and spontaneous internal lactone formation to produce ellagic acid, which probably constitutes the substrate for the formation of urolithins in bacterial metabolism. Significant amounts of urolithins D and C and trace amounts of urolithin A were detected in the jejunum of Iberian pigs fed with an ellagitannin-rich diet. Therefore, urolithin formation starts as early as in the small intestines and bacteria present in the small intestines are able to metabolize ellagitannins to a number of degradation metabolites. The fact that urolithin B was not detected in the small intestines suggests that the bacterial metabolism of ellagitannins continues in the colon and culminates with the formation of urolithin B as the final product. The distribution of urolithins in the digestive tract also points to urolithin D as the first product of microbial transformation of ellagic acid and subsequent modifications lead to intermediates with a decreasing number of phenolic hydroxy groups: urolithin C (3,7,8-trihydroxy-dibenzopyranone), urolithin A (3,8-dihydroxy-dibenzopyranone), and finally urolithin B (3-hydroxy-dibenzopyranone).

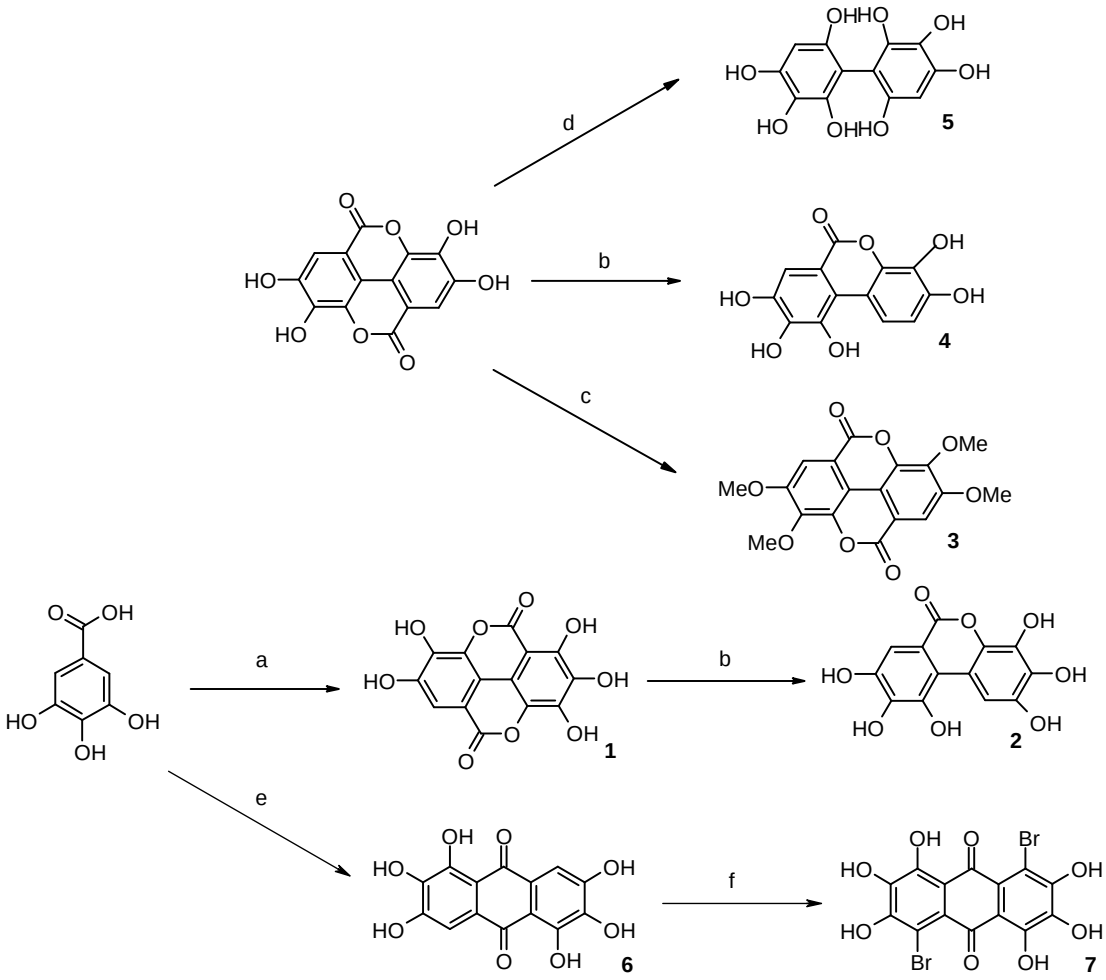
Urolithin A lacks, with respect to ellagic acid, two hydroxyl group, one at position 3 and one at position 8 and an aromatic ring. For two reasons the investigation on this natural compound is of particular importance:

- it is probably that the bioactive metabolite of ellagic acid, one of the most potent and selective inhibitors found for CK2 and consequently this family of compounds is important to understand the biological implication of CK2 inhibition.
- given the possibility to obtain a number of derivatives through suitable substitutions in opened structures, it is also possible to establish a structure-activity relationship.

For these reasons we have synthesized some derivatives of ellagic acid and urolithin A. Structure-activity relationship through docking studies on known inhibitors (TBCA, NBC, DBC) firstly suggested the introduction of small substituents to obtain an increased activity.

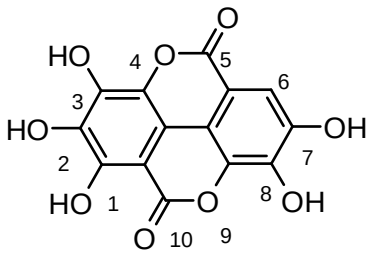
5.1.3 Chemistry

Scheme 1 here



Scheme 1. Reagents and conditions: **(a)** H_2SO_4 96%, H_2O, $K_2S_2O_8$, 4℃, overnight; **(b)** KOH, H_2O, reflux, 0.5 hour; **(c)** CH_3I/NaH, DMF, 0℃, 2 hours; **(d)** NaOH, 300℃; **(e)** H_2SO_4 96%, H_2O, 120℃, 1h, rt, 24 hours; **(f)** NBS, DMF, Br_2, rt, overnight.

1,2,3,7,8-pentahydroxy-4,9-dioxo-pyren-5,10-dione (1) Flavellagic acid



$C_{14}H_6O_9$

MW= 318,1985

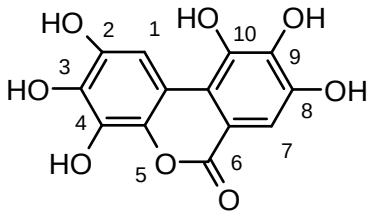
A suspension of gallic acid (10 g, 0.059 mol) in H_2SO_4 96% (80 ml) and H_2O (33 ml) was cooled at -50℃. At this temperature, to the re action mixture potassium persulfate (20 g, 0.074 mol) was added. The solution was left at 4℃ overnight. The day after a precipitate was found and the solid product was filtered and recrystallizzed by pyridine. (2.7 g, yield 14%).

^{1}H NMR [300 MHz, $(CD_3)_2SO$] δ 7.47 (s, 1H, **H6**);

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 158.71; 158.71; 151.51; 148.86; 147.49; 146.54; 139.41; 135.22; 132.02; 123.46; 122.81; 121.44; 114.87; 107.96;

HRMS calcd for $C_{14}H_5O_9$ $[M + H]^-$, 317.0142; found, 317.0031.

2,3,4,8,9,10-hexahydroxy-dibenzo[b,d]pyran-6-one (2)



$C_{13}H_8O_8$

MW= 292.2039

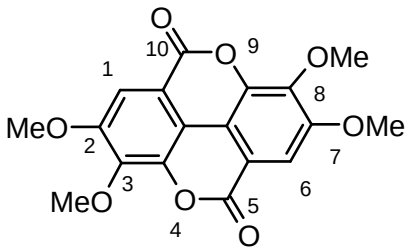
To a solution of KOH (22.5 g, 0.4 mol) in H_2O (30 ml) flavellagic acid **(1)** (3.3 g, 0.010 mol) was added. The reaction mixture was refluxed for 20 minutes and then the product was extracted from the solution with diethylether. Then the organic solvent was anhydrified by sodium sulfate and evaporated under vacuum. The residue was washed by boiling water in which the residue of Flavellagic acid **(1)** was not soluble. So flavellagic acid was filtered out and the product was recrystallized by water at room temperature. (2.1 g, yield 72%).

^{1}H NMR [300 MHz, $(CD_3)_2SO$] δ 8.02 (s, 1H, **H7**), 7.28 (s, 1H, **H1**);

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 158.73; 147.51; 146.06; 145.69; 143.64; 141.73; 135.32; 132.12; 128.36; 124.41; 115.74; 110.67; 107.76;

HRMS calcd for $C_{13}H_7O_8$ $[M + H]^-$, 291.0146; found, 291.0189.

2,3,7,8-tetramethoxy-4,9-dioxo-pyren-5,10-dione (3)



$C_{18}H_{14}O_8$

MW= 358.3075

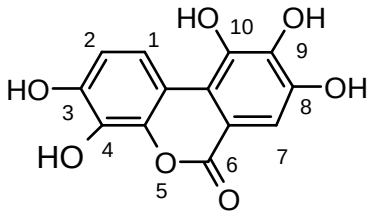
A suspension of ellagic acid (1 g, 3.31 mmol) in DMF (5 ml) was cooled at 0℃. Then to the reaction mixture NaH (0.4 g, 16.6 mmol) was added and the sospension was stirred for 20 minutes. Then CH_3I (2 ml) was added and the reaction mixture was stirred at 4℃ for 2 hours. H_2O (50 ml) was added and the product was extracted with $CHCl_3$. The product was purified by column chromatography on silica gel (eluent: $CHCl_3$) and recrystallized by a mixture of toluene:$CHCl_3$/1:1 (0.62 g, yield 52%)

^{1}H NMR [300 MHz, $(CD_3)_2SO$] δ 7.56 (s, 2H, **H1** and **H6**), 3.32 (s, 12H **CH_3**);

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 158.71; 158.71; 152.26; 152.26; 149.09; 149.09; 137.43; 137.43; 122.71; 122.71; 120.72; 120.72; 112.89; 112.89; 56.22; 56.22; 56.22; 56.22;

HRMS calcd for $C_{18}H_{15}O_8$ $[M + H]^+$, 359.0761; found, 359.0660.

3,4,8,9,10-pentahydroxy-dibenzo[b,d]pyran-6-one (4)



$C_{13}H_8O_7$

MW= 276.2045

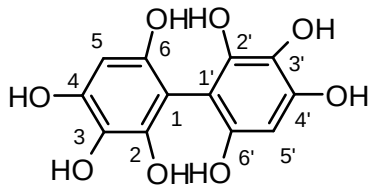
To a solution of KOH (22.5 g, 0.4 mol) in H_2O (30 ml) ellagic acid (3 g, 0.01 mol) was added. The reaction mixture was refluxed for 20 minutes and then the product was extracted from the solution with diethylether. Then the organic solvent was anhydrified by sodium sulphate and evaporated under vacuum. The residue was washed by bolling water in which the residue of ellagic acid was not soluble. So ellagic acid was filtered out and the product was recrystallized by water at room temperature. (1.7 g, yield 62%).

^{1}H NMR [300 MHz, CD_3OD] δ 8.47 (d, *J* = 8.9 Hz, 1H, **H1**), 7.41 (s, 1H, **H7**), 6.79(d, *J* = 8.9 Hz, 1H, **H2**);

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 158,87; 147.51; 146.64; 145.69; 142.24; 141.71; 139.52; 126.92; 124.46; 122.31; 115.74; 114.67; 110.66.

HRMS calcd for $C_{13}H_7O_7$ $[M + H]^-$, 275.0426; found, 275.0338.

Biphenyl-2,3,4,6,2',3',4',6'-octaol (5)



$C_{12}H_{10}O_8$

MW= 282.2087

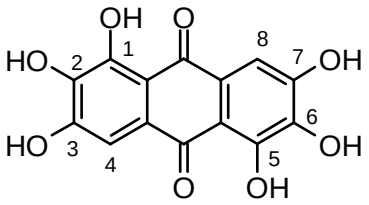
To a melted NaOH (35 g, 0.88 mol) at 300℃ ellagic acid (3 g, 0.010 mol) was added. The reaction mixture was stirred at the same temperature for 20 minutes. Then the solution was cooled at room temperature and H_2O (140 ml) was added. The insoluble residue was filtered out and the filtrate was acidified by HCl 37% until complete precipitation of the product.Then the solid was filtered and dried. (0.62 g, yield 22%).

^{1}H NMR [300 MHz, $(CD_3)_2SO$] δ 7.56 (s, 2H, **H5** and **H5'**)

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 151.18; 151.18; 149.03; 149.03; 147.11; 147.11; 129.16; 129.16; 107.09; 107.09; 98.04; 98.04;

HRMS calcd for $C_{12}H_{10}O_8$ $[M + H]^-$, 281.0661; found, 281.0560.

1,2,3,5,6,7-Hexahydroxy-anthraquinone (6)



$C_{14}H_8O_8$

MW= 304.2151

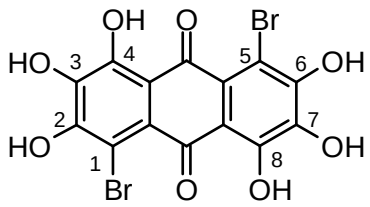
Gallic acid (8 g, 47 mmol) was solubilized in H_2SO_4 96% (22 mL) under stirring.
The reaction mixture was heated at 120℃ and it was stirred at the same temperature for one hour. Then the solution was cooled at room temperature and stirred for 24 hours. The solution was put into the ice (200 g); the day after a red precipitate was found and the solid product was filtered and dried. (4.5 g, yield 63 %)

^{1}H NMR [300 MHz, $(CD_3)_2SO$] δ 7.22 (s, 2H, **H4** and **H8**)

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 182.28; 182.28; 153.93; 153.93; 152.11; 152.11; 139.48; 139.48; 129.11; 129.11; 116.14; 116.14; 109.87; 109.87;

HRMS calcd for $C_{14}H_8O_8$ $[M + H]^-$, 303.0184; found, 303.0126.

1,5-Dibromo-2,3,4,6,7,8-hexahydroxy-anthraquinone (7)



$C_{14}H_6Br_2O_8$

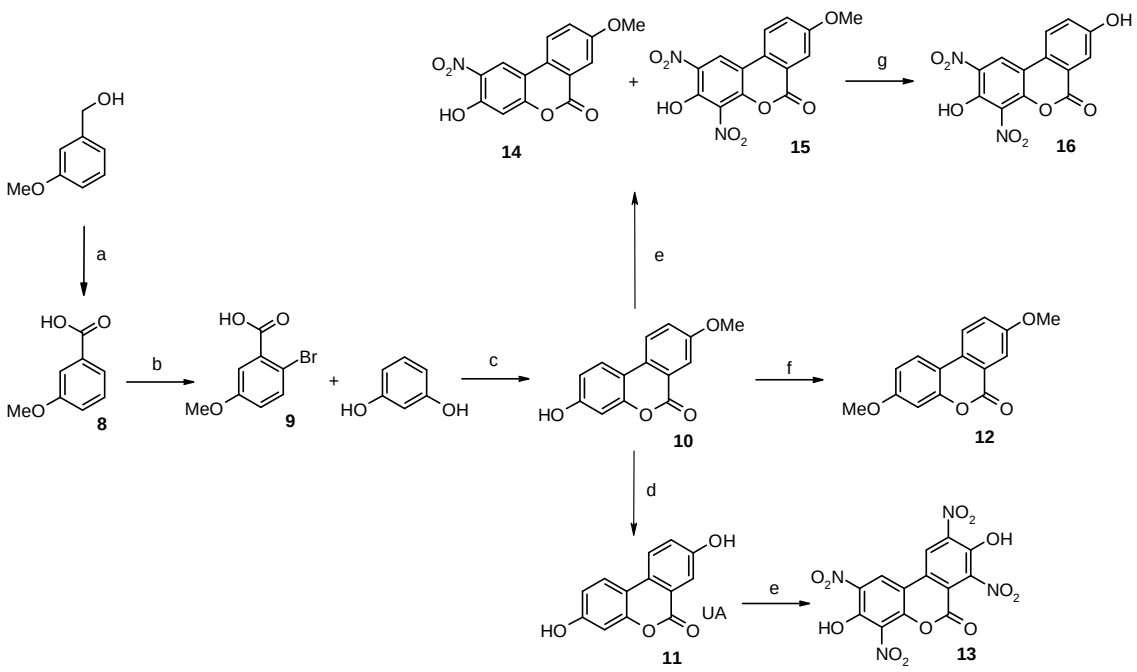
MW= 462.0071

To a solution of **(6)** (200 mg, 0,66 mmol) in DMF (9 mL) cooled at 0℃, a solution of NBS (235 mg, 1.32 mmol) in DMF (10 mL) was added dropwise.
The reaction mixture was stirred at room temperature overnight. Then the solvent was dried under vacuum. (270 mg, yield 89%)

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 182.23; 182.23; 152.96; 152.96; 148.31; 148.31; 141.64; 141.64; 128.02; 128.02; 118.31; 118.31; 111.56; 111.56;

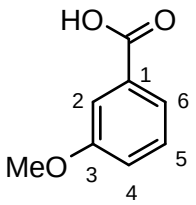
HRMS calcd for $C_{14}H_8O_8$ $[M + H]^-$, 461.0963; found, 461.0943.

Scheme 2 here



Scheme 2. Reagents and conditions: **(a)** $KMnO_4$, NaOH, reflux; **(b)** Br_2, Acetic acid, reflux; **(c)** $CuSO_4$, NaOH, reflux; **(d)** HBr, acetic acid, reflux, 11 hours; **(e)** Nitric acid 65%, acetic acid, 50℃, 4 hours; **(f)** Dimethylsulfate, K_2CO_3, aceton, reflux, 1 hours; **(g)** Pyridine hydrochloride, 210℃, 4 hours.

3-methoxy benzoic acid (8)



$C_8H_8O_3$

MW= 152.1512

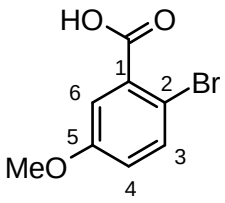
To a solution of NaOH (7.2 g, 0.18 mol) in H_2O (200 ml), 3-methoxy-benzyl-alcool (5 g, 0.036 mol) and $KMnO_4$ (9.5 g, 0.06 mol) were added. The mixture was heated at 100℃ (the solution changed its color from green to brown due to the formation of MnO_2). The warm suspension was filtered. The filtrate was acidified until complete precipitation of the product and the solid was filtered out and dried. (2.7 g, yield 49%).

^{1}H NMR [300 MHz, $CDCl_3$] δ 7.72 (ddd, *J* = 7.6 Hz, *J* = 1.5 Hz, *J* = 1.5 Hz, 1H, **H6**), 7.62-7.59 (m, 1H, **H2**), 7.39 (t, *J* = 8.0 Hz, *J* = 7.6 Hz, 1H, **H5**), 7.16-7.12 (m, 1H, **H4**), 3.77 (s, 3H, O**CH_3**);

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 169.41; 160.63; 131.35; 129.75; 122.67; 119.58; 114.53; 55.96;

HRMS calcd for $C_8H_7O_3$ $[M + H]^-$, 151.0401; found, 151.0401.

2-bromo-5-methoxy benzoic acid (9)



$C_8H_7BrO_3$

MW= 231.0472

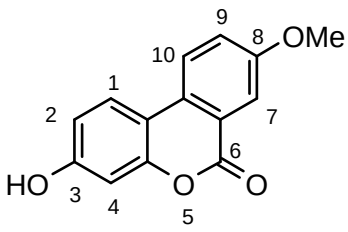
To a solution of 3-methoxy-benzoic acid **(8)** (2.7 g, 0.018 mol) in acetic acid (18 ml), a solution of bromine (2.8 g, 0.018 mol) in acetic acid (9 ml) was added dropwise. The reaction mixture was refluxed. The end of the reaction was indicated by the change of the color from orange (due to the presence of bromine) to colorless. Then the mixture was put into ice until complete precipitation of the product. The solid was filtered out, washed with a solution of water and methanol and then dried. (3.4 g, yield 82%).

^{1}H NMR [300 MHz, $CDCl_3$] δ 7.58 (d, *J* = 8.8 Hz, 1H, **H3**), 7.50 (d, *J* = 3.2 Hz, 1H, **H6**), 6.95 (dd, *J* = 8.8 Hz, *J* = 3.2 Hz, 1H, **H4**), 3.77 (s, 3H, O**CH_3**);

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 169.37; 159.69; 132.98; 132.60; 121.75; 116.73; 115.11; 55.96;

HRMS calcd for $C_8H_6BrO_3$ $[M + H]^-$, 230.9486; found, 230.9440.

3-hydroxy-8-methoxy-dibenzo[*b,d*]pyran-6-one (10)[169]



$C_{14}H_{10}O_4$

MW= 242.2334

A solution of 2-bromo-5-methoxy-benzoic acid **(9)** (3.4 g, 0.015 mol), resorcinol (3.2 g, 0.029 mol), NaOH (1.2 g, 0.029 mol) in water (15 ml) was refluxed for 20 minutes. Then a solution of $CuSO_4$ 5% (6.2 ml) was added to the reaction mixture and it was refluxed again for an hour. The end of the reaction was determined by TLC ($CHCl_3$:MeOH/9:1). The suspension obtained was filtered out and the solid residue was dried. (1.8 g, yield 51%).

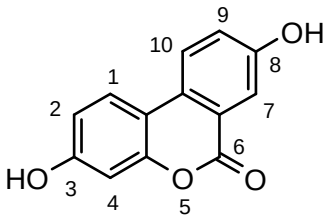
^{1}H NMR [300 MHz, $(CD_3)_2SO$] δ 10.21 (s, 1H. **OH**), 8.21 (d, *J* = 8.9 Hz, 1H, **H10**), 8.09 (d, *J* = 8.4 Hz, 1H, **H1**), 7.60 (d, *J* = 2.7 Hz, 1H, **H7**), 7.49 (dd, *J* = 8.9 Hz, *J* = 2.7 Hz, 1H, **H9**), 6.83 (dd, *J* = 8.4 Hz, *J* = 2.4 Hz, 1H, **H2**), 6.75 (d, *J* = 2.4 Hz, 1H, **H4**), 3.89 (s, 3H, O**CH_3**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 160.41; 158.83; 158.40; 151.05; 128.43; 124.07; 123.88; 123.50; 119.93; 112.99; 110.70; 109.40; 102.73; 55.45;

HRMS calcd for $C_{14}H_9O_4$ $[M + H]^-$, 241.0506; found, 241.0488.

HPLC: rt = 10.33 min, purity 98%.

3,8-dihydroxy-dibenzo[*b,d*]pyran-6-one (11) (Urolithin A)



$C_{13}H_8O_4$

MW= 228.2063

A suspension of 3-hydroxy-8-methoxy-dibenzo[*b,d*]pyran-6-one **(10)** (1.8 g, 7.4 mmol), in azeotropic mixture of bromidric acid (40 ml) and acetic acid (80 ml) was refluxed 11 hours. The end of the reaction was determined by TLC ($CHCl_3$:MeOH/9:1). The mixture was put into the ice until complete precipitation of the product. Then the solid was filtered out and dried. (1.25 g, yield 74%).

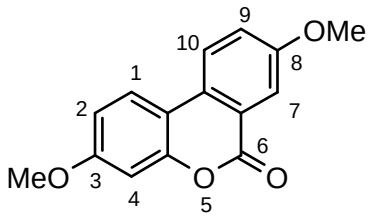
^{1}H NMR [300 MHz, $(CD_3)_2SO$] δ 8.11 (d, *J* = 8.8 Hz, 1H, **H10**), 8.02 (d, *J* = 8.7 Hz, 1H, **H1**), 7.51 (d, *J* = 2.6 Hz, 1H, **H7**), 7.32 (dd, *J* = 8.8 Hz, *J* = 2.6 Hz, 1H, **H9**), 6.81 (dd, *J* = 8.7 Hz, *J* = 2.4 Hz, 1H, **H2**), 6.72 (d, *J* = 2.4 Hz, 1H, **H4**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 160.49; 158.45; 156.86; 150.80; 126.84; 124.12; 123.94; 123.23; 120.08; 113.43; 112.50; 109.73; 102.31;

HRMS calcd for $C_{13}H_7O_4$ $[M + H]^-$, 227.0350; found, 227.0334

HPLC: rt = 8.18 min, purity >98%.

3,8-dimethoxy-dibenzo[*b,d*]pyran-6-one (12)



$C_{15}H_{12}O_4$

MW= 256.2605

K_2CO_3 (31 g, 0.225 mol) and dimethylsulfate (26.5 g, 0.21 mol) were added to a solution of 3-hydroxy-8-methoxy-dibenzo[*b,d*]pyran-6-one **(10)** (0.5 g, 2.1 mmol) in aceton (300 ml). The reaction mixture was refluxed for 40 minutes. The end of the reaction was determined by TLC ($CHCl_3$:MeOH/9:1). Then the residue of solid salt was filtered out and the solvent was evaporated under vacuum. Then the solid residue was solubilized in ethyl acetate and washed with water. The organic phase was separated, anhydrified with sodium sulphate and dried. (0.102 g, yield 19%).

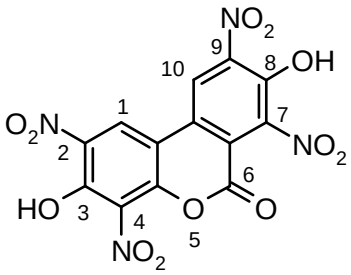
^{1}H NMR [300 MHz, $CDCl_3$] δ 7.90 (d, *J* = 8.9 Hz, 1H), 7.84 (d, *J* = 8.7 Hz, 1H), 7.74 (d, *J* = 2.8 Hz, 1H), 7.35 (dd, *J* = 8.9 Hz, *J* = 2.8 Hz, 1H), 6.88 (dd, *J* = 8.7 Hz, *J* = 2.6 Hz, 1H), 6.84 (d, *J* = 2.6 Hz, 1H), 3.95 (s, 3H), 3.87 (s, 3H);

^{13}C NMR [300 MHz, $CDCl_3$] δ 161.60; 160.68; 159.17; 151.67; 128.61; 124.49; 123.14; 122.81; 121.00; 112.39; 111.32; 110.99; 101.88; 55.69; 55.69;

HRMS calcd for $C_{15}H_{13}O_4$ $[M + H]^+$, 257.0808; found, 257.0790

HPLC: rt = 12.96 min, purity 98%.

3,8-dihydroxy-2,4,7,9-tetranitro-6*H*-dibenzo[*b,d*]pyran-6-one (13)



$C_{13}H_4N_4O_{12}$

MW= 408.1964

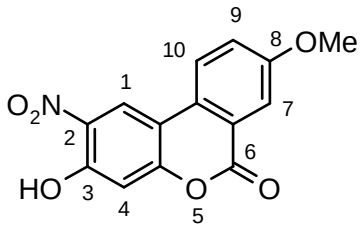
To a solution of 3,8-dihydroxy-dibenzo[*b,d*]pyran-6-one **(11)** (0.5 g, 2.2 mmol) in acetic acid (25 ml), nitric acid 65% (0.83 g, 13.2 mmol) was added. The reaction mixture was heated at 50℃ for 4 hours. The end of the reaction was determined by TLC (EtOAc:nHexane:MeOH/7:2:1). Then the solvent was evaporated under vacuum and the residue was recrystallized by acetic acid. (0.2 g, yield 22%).

^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 9.51 (s, 1H, **H10**), 9.49 (s, 1H, **H1**);

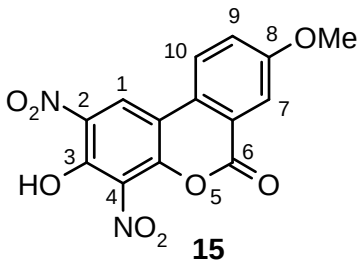
^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 154.85; 149.73; 149.00; 147.71; 142.63; 134.36; 126.81; 125.66; 124.53; 123.52; 122.39; 118.98; 112.03;

HRMS calcd for $C_{13}H_3N_4O_{12}$ $[M + H]^-$, 407.0107; found, 407.0173.

3-hydroxy-8-methoxy-2-nitro-6*H*-dibenzo[*b,d*]pyran-6-one (14) and **3-hydroxy-8-methoxy-2,4-dinitro-6*H*-dibenzo[*b,d*]pyran-6-one (15)**



14



15

$C_{14}H_9NO_6$
MW= 287.2309

$C_{14}H_8N_2O_8$
MW= 332.2285

To a solution of 3-hydroxy-8-methoxy-dibenzo[*b,d*]pyran-6-one **(10)** (0.5 g, 2.1 mmol) in acetic acid (12 ml), nitric acid 65% (0.27 g, 4.3 mmol) was added. The reaction mixture was heated at 50℃ for 4 hours. The end of the reac tion was determined by TLC ($CHCl_3$:MeOH/9:1). The suspension obtained was filtered out and the solid residue was dried. The solid residue was composed by two compouds: 3-hydroxy-8-methoxy-2-nitro-6*H*-dibenzo[*b,d*]pyran-6-one **(14)** and 3-hydroxy-8-methoxy-2,4-dinitro-6*H*-dibenzo[*b,d*]pyran-6-one **(15)** which were separated by a column chromatography on silica gel ($CHCl_3$:MeOH/9:1).

Compound **(14)** (0.078 g, yield 13%).

^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 8.81 (s, 1H, **H1**), 8.37 (d, *J* = 8.9 Hz, 1H, **H10**), 7.62 (d, *J* = 2.8 Hz, 1H, **H7**), 7.53 (dd, *J* = 8.9 Hz, *J* = 2.8 Hz, 1H, **H9**), 7.03 (s, 1H, **H4**), 4.01 (s, 3H, O**CH$_3$**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 161.73; 161.62; 155.65; 155.00; 137,30; 128.91; 128.69; 126.01; 122.99; 122.75; 113.45; 112.47; 108.01; 57.91;

HRMS calcd for $C_{14}H_8NO_6$ $[M + H]^-$, 286.0357; found, 286.0383.

HPLC: rt = 12.28 min, purity 98%.

Compound **(15)** (0.41g, yield 59%).

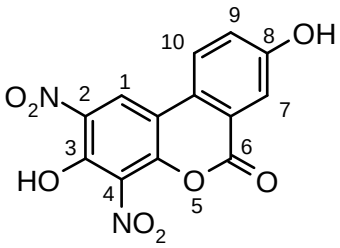
^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 9.05 (s, 1H, **H1**), 8.45 (d, *J* = 8.9 Hz, 1H, **H10**), 7.72 (d, *J* = 2.8 Hz, 1H, **H7**), 7.59 (dd, *J* = 8.9 Hz, *J* = 2.8 Hz, 1H, **H9**), 4.01 (s, 3H, O**CH$_3$**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 171.94; 159.23; 158.22; 151.56; 145.43; 135.78; 126.63; 124.45; 124.10; 121.25; 119.93; 111.34; 106.41; 55.62;

HRMS calcd for $C_{14}H_7N_2O_8$ $[M + H]^-$, 331.0208; found, 331.0195.

HPLC: rt = 11.48 min, purity 98%.

3,8-dihydroxy-2,4-dinitro-6*H*-dibenzo[*b,d*]pyran-6-one (16)



$C_{13}H_6N_2O_8$

MW= 318.2014

In a flask of 50 ml, pyridine chlorohydrate (in surplus) and 3-hydroxy-8-methoxy-2,4-dinitro-6*H*-dibenzo[*b,d*]pyran-6-one (0.2 g, 0.6 mmol) **(15)** were put. The reaction mixture was heated until 210℃ to obtain the comple te solubilization of pyridine, and it was stirred for 4 hours. Then the reaction mixture was cooled. During the decreasing of temperature the pyridine chlorohydrate returned to solid phase. Then ethyl acetate was added to the sospension and the reaction mixture was washed with water three times (to solubilized all residue of pyridine chlorohydrate). The organic phase was separated, anhydrified by sodium sulphate and dried. The solid residue was purified by column chromatography on silica gel ($CHCl_3$:MeOH/9:1) (60 mg, yield 31%).

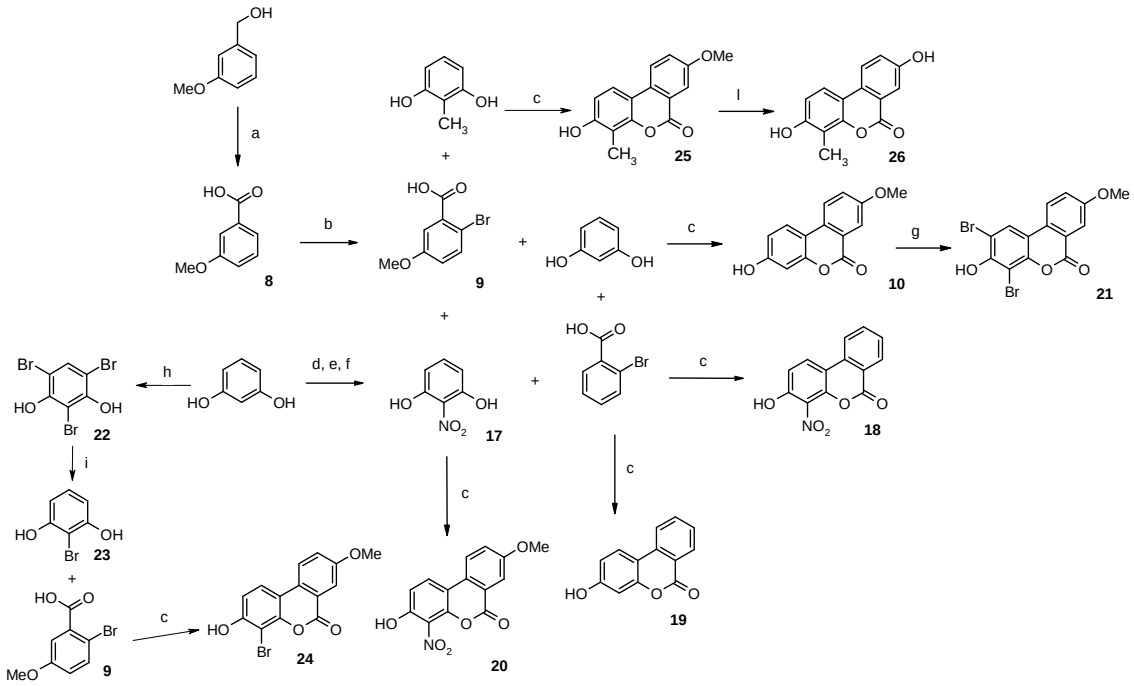
^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 8.96 (s, 1H, **H1**), 8.56 (d, *J* = 8.9 Hz, 1H, **H10**), 7.69 (d, *J* = 2.8 Hz, 1H, **H7**), 7.43 (dd, *J* = 8.9 Hz, *J* = 2.8 Hz, 1H, **H9**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 158.73; 157.36; 154.67; 149.41; 135.22; 131.12; 130.45; 129.96; 129.25; 127.60; 127.35; 121.68; 116.63;

HRMS calcd for $C_{13}H_5N_2O_8$ $[M + H]^-$, 317.2294; found, 317.2369.

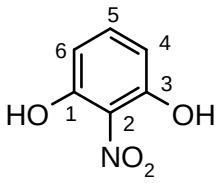
HPLC: rt = 9.92 min, purity 99%.

Scheme 3 here



Scheme 3. Reagents and conditions: **(a)** $KMnO_4$, NaOH, reflux; **(b)** Br_2, Acetic acid, reflux; **(c)** $CuSO_4$, NaOH, reflux; **(d)** H_2SO_4; **(e)** HNO_3, H_2SO_4, r.t. 1 hour ; **(f)** H_2O, reflux. **(g)** NBS, CCl_4, reflux, 2 hours; **(h)** Br_2, $CHCl_3$, reflux, 2 hours; **(h)** Na_2SO_3, H_2O, reflux 12 hours; **(l)** HBr, acetic acid, reflux, 11 hours.

2-nitro-resorcinol (17)



$C_6H_5NO_4$

MW= 155.1111

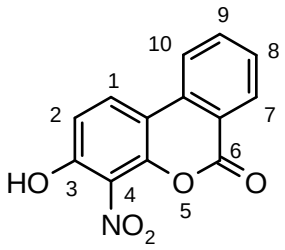
Resorcinol (5 g, 0.05 mol) was solubilized in 36 ml of hot H_2SO_4 96% (33.84 g, 0.35 mol). After 5 minutes the resorcidin-sulfonic acid began to precipitate. The suspension was cooled at room temperature with the consequent formation of a white precipitate. At this suspension HNO_3 65% (2.84 g, 0.05 mol) and H_2SO_4 (9.05 g, 0.09 mol) were added under strong stirring until a complete solubilization. Then the stirring was suspended and ice (60g/100g of solution) was added slowly. The reaction mixture was refluxed and the product was obtained by distillation. The residue was purified by crystallization by absolute ethanol to obtain a red needles (3.8 g, yield 60%).

^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 7.40 (t, *J* = 8.4 Hz, 1H, **H5**), 6.61 (d, *J* = 8.4 Hz, 2H,**H4** and **H6**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 154.57; 154.57; 137.76; 122.86; 109.49; 109.49;

HRMS calcd for $C_6H_4NO_4$ $[M + H]^-$, 154.0146; found, 154.0157.

3-hydroxy-4-nitro-dibenzo[*b,d*]pyran-6-one (18)



$C_{13}H_7NO_5$

MW= 257.2044

A solution of 2-bromo-benzoic acid (1 g, 0.005 mol), 2-nitro-resorcinol (1 g, 0.006 mol), NaOH (0.4 g, 0.010 mol) in water (20 ml) was refluxed for 30 minutes Then a solution of $CuSO_4$ 10% (0.5 ml) was added to the reaction mixture and it was refluxed again for one hour. The end of the reaction was determined by TLC ($CHCl_3$:Aceton/1:1). The suspension obtained was filtered out and the solid residue was dried. The compound was purified by column chromatography on silica gel ($CHCl_3$:MeOH/9:1) (0.2 g, yield 16%).

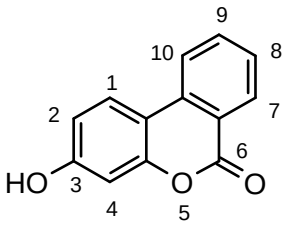
^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 8.16 (dd, *J* = 8.9 Hz, *J* = 2.7 Hz, 1H, **H7**), 7.82 (d, *J* = 9.0 Hz, 1H, **H1**), 7.53-7-50 (m, 2H, **H9**, **H10**), 7.37-7.32 (m, 1H, **H8**), 6.96 (d, *J* = 9.0 Hz, 1H, **H2**)

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 160.71; 154.18; 145.68; 137.34; 136.31; 132.02; 130.92; 130.32; 127.36; 123.71; 121.24; 115.67; 111.66;

HRMS calcd for $C_{13}H_6NO_5$ $[M + H]^-$, 256.0251; found, 256.0130.

HPLC: rt = 10.16 min, purity >98%.

3-hydroxy-dibenzo[*b,d*]pyran-6-one (19) Urolithin B



$C_{13}H_8O_3$

MW= 212.2069

A solution of 2-bromo-benzoic acid (2 g, 0.010 mol), resorcinol (2 g, 0.018 mol), NaOH (0.4 g, 0.010 mol) in water (20 ml) was refluxed for 30 minutes Then a solution of $CuSO_4$ 10% (0.5 ml) was added to the reaction mixture and it was refluxed again for one hour. The end of the reaction was determined by TLC ($CHCl_3$:Aceton/1:1). The suspension obtained was filtered out and the solid residue was dried. The compound was purified by column chromatography on silica gel ($CHCl_3$:MeOH/9:1) (740 mg, yield 35%).

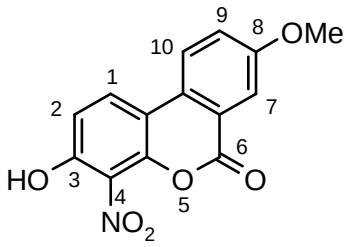
^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 8.20 (dd, *J* = 8.9 Hz, *J* = 2.7 Hz, 1H, **H7**), 7.63-7.57(m, 2H, **H10**, **H9**) 7.47-7.42 (m, 2H, **H1**, **H8**), 6.69 (dd, *J* = 9.0 Hz, *J* = 2.1 Hz 1H, **H2**), 6.67 (d, *J* = 2.1 Hz, 1H, **H4**)

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 160.73; 157.89; 153.01; 135.56; 134.57; 130.82; 129.76; 129.04; 127.85; 127.65; 125.03; 113.22; 109.84;

HRMS calcd for $C_{13}H_7O_3$ $[M + H]^-$, 211.4534; found, 211.4237

HPLC: rt = 9.87 min, purity 98%.

3-hydroxy-4-nitro-8-methoxy-dibenzo[*b,d*]pyran-6-one (20)



$C_{14}H_9NO_6$

MW= 287.2309

A solution of 2-bromo-5-methoxy-benzoic acid (1g, 0.004 mol), 2-nitro-resorcinol (0.8 g, 0.005 mol), NaOH (0.4 g, 0.010 mol) in water (20 ml) was refluxed for 30 minutes. Then a solution of $CuSO_4$ 10% (0.5 ml) was added to the reaction mixture and it was refluxed again for one hour. The end of the reaction was determined by TLC ($CHCl_3$:Aceton/1:1). The suspension obtained was filtered out and the solid residue was dried. The compound was purified by column chromatography on silica gel ($CHCl_3$:MeOH/8:2) (600 mg, yield 52%).

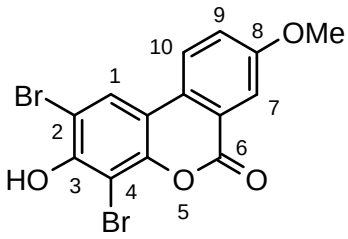
^{1}H NMR [300 MHz, $(CD_3)_2SO$] δ 8.29 (d, *J* = 8.8 Hz, 1H, **H1**), 8.26 (d, *J* = 8.8 Hz, 1H, **H10**), 7.62 (d, *J* = 2.9 Hz, 1H, **H7**), 7.55 (dd, *J* = 8.8 Hz, *J* = 2.9 Hz, 1H, **H9**), 7.06 (d, *J* = 8.8 Hz, 1H, **H2**), 3.91 (s, 3H, O**CH_3**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 159.18; 158.68; 150.28; 148.54; 141.97; 127.13; 125.43; 124.15; 124.08; 119.99; 113.36; 111.14; 109.84; 55.65

HRMS calcd for $C_{14}H_9NO_6$ $[M + H]^-$, 286.0984; found, 286.0367

HPLC: rt = 10.88 min, purity >99%.

2,4-dibromo-3-hydroxy-8-methoxy-6*H*-dibenzo[*b,d*]pyran-6-one (21)



$C_{14}H_8Br_2O_4$

MW= 400.0255

To a solution of 3-hydroxy-8-methoxy-dibenzo[*b,d*]pyran-6-one **(10)** (1 g, 4.1 mmol) in CCl_4 (40 ml), NBS (0.73 g, 4.1 mmol) and benzoyl peroxide (radicalic starter) (10 mg) were added. The reaction mixture was refluxed for 2 hours. The end of the reaction was determined by TLC (nHexane:EtOAc/8:2). The suspension obtained was filtered out and the solid residue was dried. The solid residue was purified by a column cromatography on silica gel ($CHCl_3$:EtOAc/9:1) (0.078 g, yield 30%).

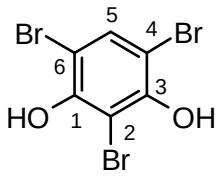
^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 8.11 (s, 1H, **H1**), 7.90 (d, *J* = 8.9 Hz, 1H, **H10**), 7.70 (d, *J* = 2.8 Hz, 1H, **H7**), 7.42 (dd, *J* = 8.9 Hz, *J* = 2.8 Hz, 1H, **H9**), 3.95 (s, 3H, O**CH$_3$**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 159.53; 158.75; 155.73; 151.83; 134.36; 130.05; 129.96; 128.84; 127.35; 120.04; 115.02; 114.62; 113.37; 55.96;

HRMS calcd for $C_{14}H_7Br_2O_4$ $[M + H]^-$, 399.0453; found, 399.0857.

HPLC: rt = 12.24 min, purity 98%.

2,4,6-tribromo-resorcinol (22)



$C_6H_3Br_3O_2$

MW= 346.8016

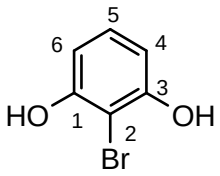
Bromine (4.5ml, 14 g, 0.087 mol) was added dropwise to a sospension of resorcinol (3 g, 0.027 mol) in $CHCl_3$. The reaction mixture was refluxed for 2 hours. The end of the reaction was determined by TLC ($CHCl_3$:MeOH/9:1). Then the solvent was partially evaporated under vacuum and the reaction mixture was put at 4℃ to crystallize. Finally the crystals obtained were filtered out and they were dried (5.4 g, yield 58%).

^{1}H NMR [300 MHz, ($CDCl_3$] δ 7.60 (s, 1H, **H5**), 5.92 (s, 2H,**OH**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 152.96; 152.96; 136.63; 110.82; 110.82; 110.65;

HRMS calcd for $C_6H_2Br_3O_2$ $[M + H]^-$, 345.4014; found, 345.4057.

2-bromo-resorcinol (23)



$C_6H_5BrO_2$

MW= 189.0095

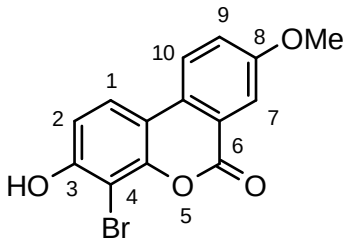
To a suspension of 2,4,6-tribromo-resorcinol (2 g, 0.0058 mol) in H_2O (40 ml), a solution of Na_2SO_3 (21.8 g, 0.173 mol) in H_2O (60ml) was added. The reaction mixture was acidified by H_2SO_4 until pH = 2 and then it was refluxed for 12 hours. The end of the reaction was determined by TLC ($CHCl_3$:MeOH/9:1). The reaction mixture was cooled, the acidity was neutralized by $NaHCO_3$, and the product was extracted from water by toluene. The organic phase was separated, anhydrified by sodium sulphate and dried. (0.702 g, yield 64%).

^{1}H NMR [300 MHz, ($CDCl_3$] δ 6.94 (t, *J* = 8.1 Hz, 1H, **H5**), 6.39 (d, *J* = 8.1 Hz, 2H,**H4**, **H6**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 158.11; 158.11; 130.63; 110.72; 110.72; 106.25;

HRMS calcd for $C_6H_4BrO_2$ $[M + H]^-$, 188.0014; found, 188.0072.

4-bromo-3-hydroxy-8-methoxy-dibenzo[b,d]pyran-6-one (24)



$C_{14}H_9BrO_4$

MW= 321.1294

A solution of 2-bromo-5-methoxy-benzoic acid (200 mg, 0.866 mmol), 2-nitro-resorcinol (327 mg, 1.732 mmol), NaOH (69 mg, 1.732 mmol) in water (10 ml) was refluxed for 30 minutes Then a solution of $CuSO_4$ 10% (0.5 ml) was added to the reaction mixture and it was refluxed again for one hour. The end of the reaction was determined by TLC ($CHCl_3$:MeOH/9:1). The reaction mixture was cooled, the precipitate was filtered out and the solid residue was dried. The compound was purified by column chromatography on silica gel ($CHCl_3$:MeOH/95:5) (81 mg, yield 29%).

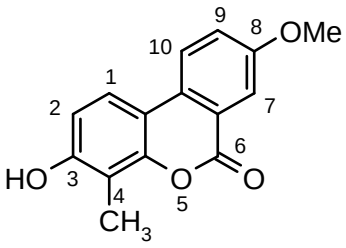
^{1}H NMR [300 MHz, ($CDCl_3$)] δ 7.93 (d, *J* = 8.9 Hz, 1H, **H1**), 7.84 (d, *J* = 8.8 Hz, 1H, **H10**), 7.79 (d, *J* = 2.8 Hz, 1H, **H7**), 7.40 (dd, *J* = 8.8 Hz, *J* = 2.8 Hz, 1H, **H9**), 7.05 (d, *J* = 8.8 Hz, 1H, **H2**), 5.89 (s br, 1H, **OH**), 3.94 (s, 3H, O**CH_3**);

^{13}C NMR [300 MHz, $(CD_3)_2SO$] δ 159.53; 158.74; 156.71; 156.02; 130.07; 128.84; 128.79; 127.78; 127.35; 120.04; 115.43; 115.09; 112.43; 55.97

HRMS calcd for $C_{14}H_8BrO_4$ $[M + H]^-$, 320.1454; found, 320.1398

HPLC: rt = 11.08 min, purity >99%.

3-hydroxy-8-methoxy-4-methyl-dibenzo[b,d]pyran-6-one (25)



$C_{15}H_{12}O_4$

MW= 256.2605

A solution of 2-bromo-5-methoxy-benzoic acid (280 mg, 1.206 mmol), 2-methyl-resorcinol (300 mg, 2.416 mmol), NaOH (96 g, 2.4 mmol) in water (10 ml) was refluxed for 30 minutes Then a solution of $CuSO_4$ 10% (0.5 ml) was added to the reaction mixture and it was refluxed again for one hour. The end of the reaction was determined by TLC ($CHCl_3$:MeOH/9:1). The reaction mixture was cooled and the suspension obtained was filtered out and the solid residue was dried. The compound was purified by column chromatography on silica gel ($CHCl_3$:MeOH/9:1) (100 mg, yield 32%).

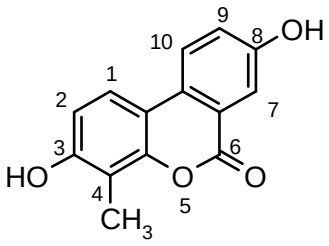
^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 8.16 (d, *J* = 8.9 Hz, 1H, **H10**), 7.89 (d, *J* = 8.7 Hz, 1H, **H1**), 7.70 (d, *J* = 2.8 Hz, 1H, **H7**), 7.45 (dd, *J* = 8.9 Hz, *J* = 2.8 Hz, 1H, **H9**), 6.94 (d, *J* = 8.7 Hz, 1H, **H2**), 3.85 (s, 3H, O**CH_3**), 2.31 (s, 3H, **CH_3**);

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 159.52; 158.71; 155.53; 154.13; 130.07; 128.83; 127.34; 126.73; 125.45; 121.22; 120.08; 115.03; 113.12; 55.96, 4.43;

HRMS calcd for $C_{15}H_{11}O_4$ $[M + H]^-$, 255.0663; found, 255.0581

HPLC: rt = 11.00 min, purity 98%.

3,8-dihydroxy-4-methyl-6*H*-dibenzo[*b,d*]pyran-6-one (26)



$C_{14}H_{10}O_4$

MW= 242.2268

A suspension of 3-hydroxy-8-methoxy-4-methyl-dibenzo[*b,d*]pyran-6-one **(25)** (0.1 g, 0.39 mmol), in azeotropic mixture of bromidric acid (4 ml) and acetic acid (8 ml) was refluxed 11 hours The end of the reaction was determined by TLC ($CHCl_3$:EtOAc/7:3). The mixture was put into the ice until complete precipitation of the product. Then the solid was filtered out and purified by column chromatography on silica gel ($CHCl_3$:EtOAc/7:3) (53 g, yield 56%).

^{1}H NMR [300 MHz, $(CD_3)_2CO$] δ 8.96 (s, 1H, **H1**), 8.56 (d, *J* = 8.9 Hz, 1H, **H10**), 7.69 (d, *J* = 2.8 Hz, 1H, **H7**), 7.43 (dd, *J* = 8.9 Hz, *J* = 2.8 Hz, 1H, **H9**);

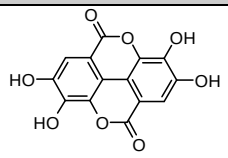
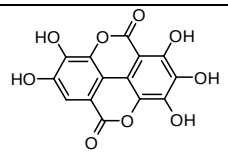
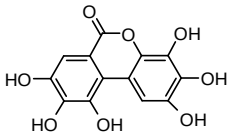
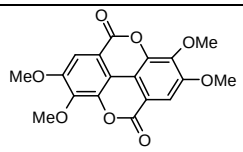
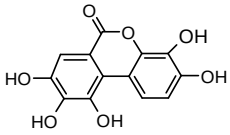
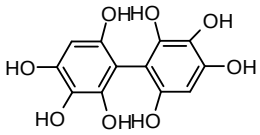
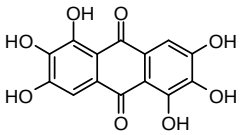
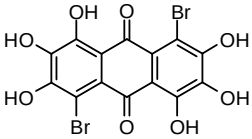
^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 158.72; 157.34; 155.56; 154.29; 132.43; 129.28; 127.64; 126.72; 125.46; 121.68; 121.29; 116.64; 113.18; 4.49;

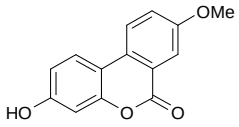
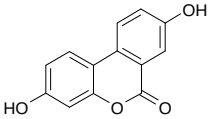
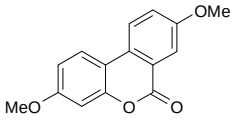
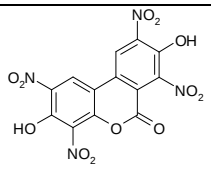
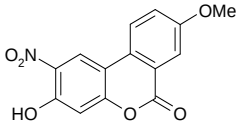
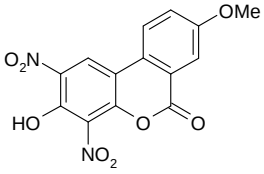
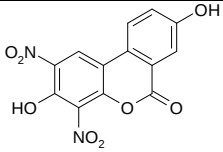
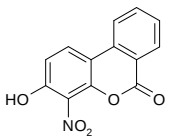
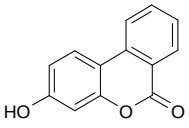
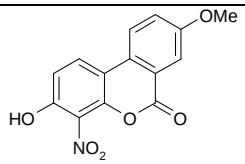
HRMS calcd for $C_{14}H_{11}O_4$ $[M + H]^+$, 243.0663; found, 243.0629.

HPLC: rt = 10.37 min, purity >98%.

5.1.4 BIOLOGICAL RESULTS

All the biological results are reported in the following table.

	Cas Name	Structure	nCK2 a 20 μM [ATP]
Ellagic acid	2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione		0,04μM
1	1,2,3,7,8-pentahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione		2,10 μM
2	2,3,4,8,9,10-hexahydroxy-benzo[c]chromen-6-one		2,20μM
3	2,3,7,8-tetramethoxy-chromeno[5,4,3-cde]chromene-5,10-dione		>40
4	3,4,8,9,10-pentahydroxy-benzo[c]chromen-6-one		0,20μM
5	Biphenyl-2,3,4,6,2',3',4',6'-octaol		0,36μM
6	1,2,3,5,6,7-hexahydroxy-anthraquinone		0,66μM
7	1,5-dibromo-2,3,4,6,7,8-hexahydroxy-anthraquinone		0,25μM

10	3-hydroxy-8-methoxy-benzo[c]chromen -6-one		3,47µM
11	3,8-dihydroxy-benzo[c]chromen-6-one		0,39µM
12	3,8-dimethoxy-benzo[c]chromen-6-one		>40
13	3,8-dihydroxy-2,4,7,9-tetranitro-benzo[c]chromen-6-one		>40
14	3-hydroxy-8-methoxy-2-nitro-benzo[c]chromen -6-one		2µM
15	3-hydroxy-8-methoxy-2,4-dinitro-benzo[c]chromen -6-one		3,05µM
16	3,8-dihydroxy-2,4-dinitro-benzo[c]chromen-6-one		0,6µM
18	3-hydroxy-4-nitro-benzo[c]chromen-6-one		0,3µM
19	3-hydroxy-benzo[c]chromen-6-one		6,5µM
20	3-hydroxy-8-methoxy-4-nitro-benzo[c]chromen -6-one		0,026µM

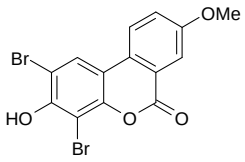
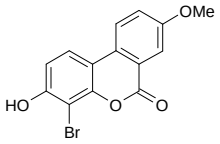
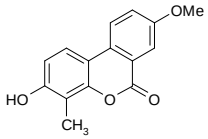
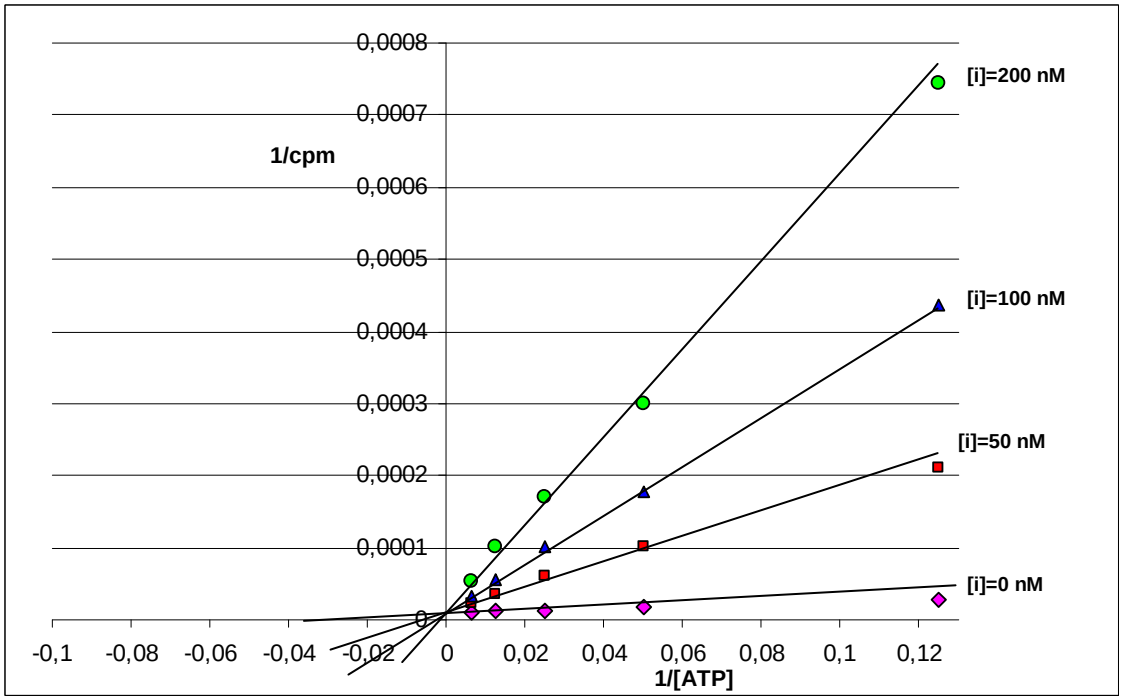
21	3-hydroxy-8-methoxy-2,4-dibromo-benzo[c]chromen-6-one		0,3µM
24	3-hydroxy-8-methoxy-4-bromo-benzo[c]chromen-6-one		0,015µM
25	3-hydroxy-8-methoxy-4-methyl-benzo[c]chromen-6-one		2,5µM

Table6: Biological results of derivatives of ellagic acid and Urolithin

Compounds 20 and 24 have been demonstrated very potent inhibitors of CK2. For compound 24 the K_i value has been determined and confirms its ATP mimetic action (see figure 25).



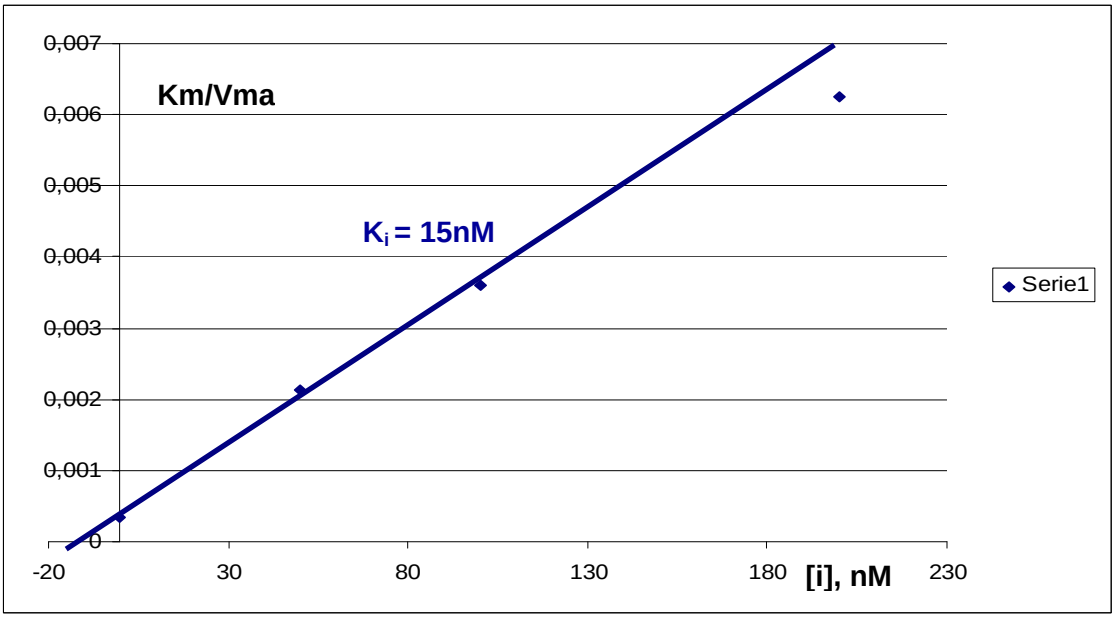


Figure 25: Ki of compound 24 for nCK2

Moreover the selectivity of this compound on a in house kinase panel has been performed.

From the results reported in table7 we can see that compound 24 is a quite selective inhibitor towards CK2.

	nCK2 at 20 µM [ATP]	**PIM1** at 100 µM [ATP]	**HIPK2** at 20 µM [ATP]	**PKA** at 20 µM [ATP]	**AURORA A** at 20 µM [ATP]	**DIRK 1a** at 20 µM [ATP]	**GCK** at 20 µM [ATP]	**nCK1** at 20 µM [ATP]	**GSK3β** at 20 µM [ATP]
Compound 24	0,015µM	1,8µM	13,42µM	>40	39µM	0.1µM	>40µM	27µM	>40µM

Table7: Partial selectivity screening of compound 24

5.2 TETRAIODOBENZIMIDAZOLE DERIVATIVES

The work described in this part is focused on some poly-iodobenzimidazole derivatives. Searching for CK2 inhibitors, just in the late 1980s polyhalogenated benzimidazoles were found to represent a valuable scaffold to effectively compete with ATP binding[92] and 4,5,6,7-tetrabromobenzotriazole (TBB) was later demonstrated to be one of the most powerful and selective cell permeable inhibitors of CK2[121,170] The presence of four bromine atoms on the benzene ring of TBB is critical to fill the CK2 hydrophobic pocket adjacent to the ATP-binding site and to assure optimal apolar interactions with some bulky side chains, in particular those of Val66 and Ile174[171,172]

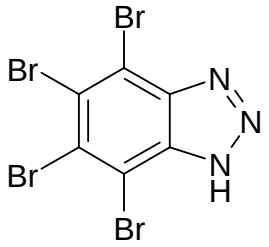
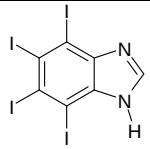
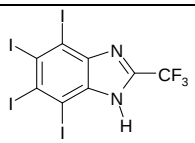
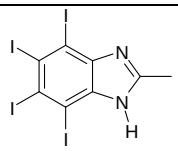
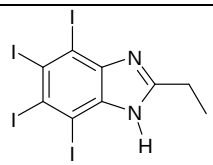
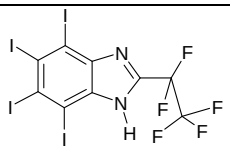
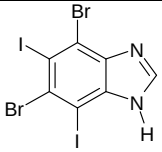
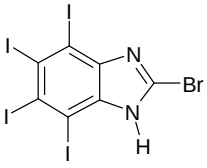


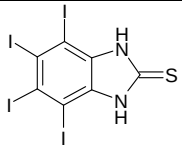
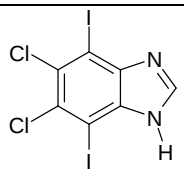
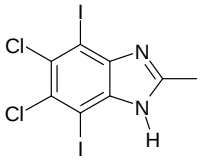
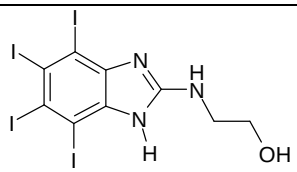
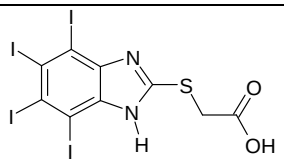
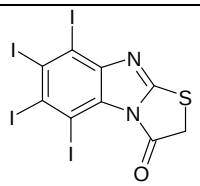
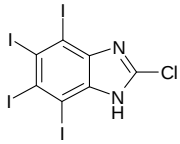
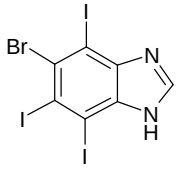
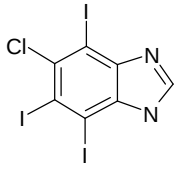
Figure 26: Chemical structure of TBB

A direct correlation was also recently established for TBB derivatives between the log K_i and the variation in the accessible surface area upon binding on CK2[173]. From this point of view, it is well known that the reduction of the number of bromine atoms or their replacement by less bulky halogens like chlorine and fluorine severely impairs the inhibitory efficiency[92,105]. However, the replacement of all four bromine atoms of the benzimidazole or benzotriazole moiety by bulkier iodines was hard to obtain and iodo-bromo or iodo-chloro benzimidazoles were unknown. Recently, structurally related tetraiodinated-isoindole derivatives were synthesized and found to inhibit protein kinase CK2 in an ATP-competitive manner with IC_{50} values between 0.15 and 1.5 μM[173]. The highest efficiency documented in literature for a CK2 inhibitor belongs to a class of pyrazolo-triazine derivatives reported to affect CK2 with Ki values in the nanomolar and subnanomolar range[134,174]. Regrettably, however, the experimental conditions for these assays were not detailed; this hampers reliable comparison with other inhibitors.

5.2.1 NEW TETRAIODOBENZIMIDAZOLE DERIVATIVES

In this part of work the structure–activity analysis has been performed in comparison with the corresponding tetrabrominated derivatives. The synthesis of a number of tetraiodinatedbenzimidazoles has been done in the laboratory of Professor Zygmunt Kazimierczuk (Warsaw, Poland) [175]. (Table 8 and 9)

	Structure	Name
K88 (TIBI)		4,5,6,7-tetraiodo-*1H*-benzimidazole
K89		4,5,6,7-tetraiodo-2-trifluoromethyl-*1H*-benzimidazole
K92		4,5,6,7-tetraiodo-2-methyl-*1H*-benzimidazole
K93		2-ethyl-4,5,6,7-tetraiodo-*1H*-benzimidazole
K94		4,5,6,7-tetraiodo-2-pentafluoroethyl-*1H*-benzimidazole
K95		4,6-dibromo-5,7-diiodo-*1H*-benzimidazole
K96		2-bromo-4,5,6,7-tetraiodo-*1H*- benzimidazole

K97		4,5,6,7-tetraiodo-1,3-dihydro-benzimidazole-2-thione
K98		5,6-dichloro-4,7-diiodo-*1H*-benzimidazole
K99		5,6-dichloro-4,7-diiodo-2-methyl-*1H*-benzimidazole
K100		2-[(2-hydroxyethyl)amino]-4,5,6,7-tetraiodo-*1H*-benzimidazole
K101		4,5,6,7-tetraiodo-*1H*-benzimidazol-2-ylsulfanyl)-acetic acid
K102		5,6,7,8-tetraiodo-benzo[4,5]imidazo [2,1-b]thiazol-3-one
K103		2-chloro-4,5,6,7-tetraiodo-*1H*-benzimidazole
K104		5-bromo-4,6,7-triiodo-*1H*-benzimidazole
K105		5-chloro-4,6,7-triiodo-*1H*-benzimidazole

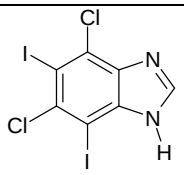
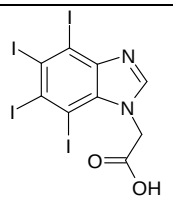
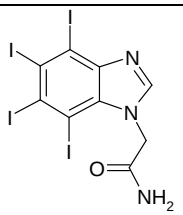
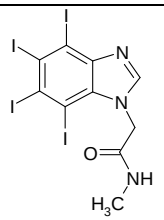
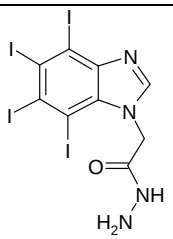
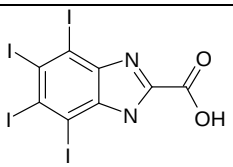
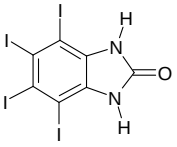
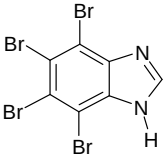
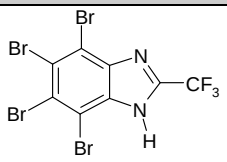
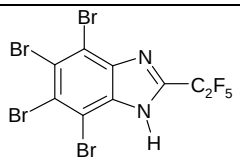
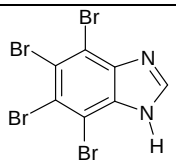
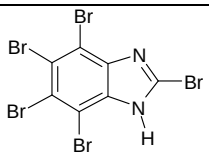
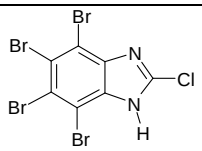
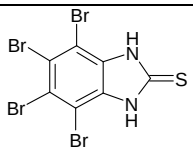
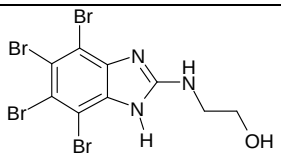
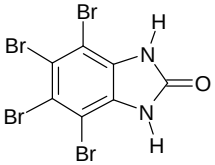
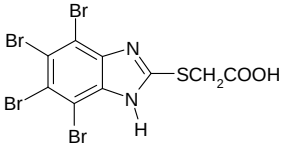
K106		4,6-dichloro-5,7-diiodo-*1H*-benzimidazole
K107		(4,5,6,7-tetraiodo-benzimidazol-*1H*-yl)-acetic acid
K108		2-(4,5,6,7-tetraiodo-benzimidazol-*1H*-yl)-acetamide
K109		N-methyl-2-(4,5,6,7-tetraiodo-benzimidazol-*1H*-yl)-acetamide
K110		(4,5,6,7-tetraiodo-benzimidazol-*1H*-yl)-acetic acid hydrazide
K111		4,5,6,7-Tetraiodo-*1H*-benzimidazole-2-carboxylic acid
K112		4,5,6,7-tetraiodo-1,3-dihydro-benzimidazol-2-one
K113 (TCI)		4,5,6,7-tetrachloro-*1H*-benzimidazole

Table8: Tetraiodo and other tetrahalogen-benzimidazole derivatives

	Structure	Name
K10		4,5,6,7-tetrabromo-2-trifluoromethyl-*1H*-benzimidazole
K11		4,5,6,7-tetrabromo-2-pentafluoroethyl-*1H*-benzimidazole
K17 (TBI)		4,5,6,7-tetrabromo-*1H*-benzimidazole
K20		2-bromo-4,5,6,7-tetrabromo-*1H*-benzimidazole
K21		2-chloro-4,5,6,7-tetrabromo-*1H*-benzimidazole
K22		4,5,6,7-tetrabromo-1,3-dihydro-benzimidazole-2-thione
K30		2-[(2-hydroxyethyl)amino]-4,5,6,7-tetrabromo-*1H*-benzimidazole
K32		4,5,6,7-tetrabromo-1,3-dihydro-benzimidazol-2-one
K33		4,5,6,7-tetrabromo-*1H*-benzimidazol-2-ylsulfanyl)-acetic acid

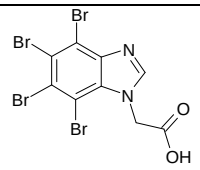
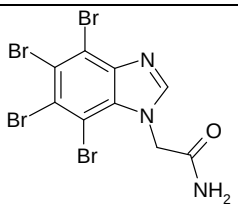
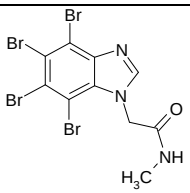
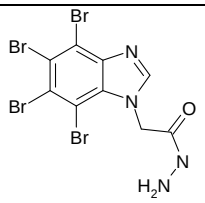
K68		(4,5,6,7-tetrabromo-benzimidazol-*1H*-yl)-acetic acid
K83		2-(4,5,6,7-tetrabromo-benzimidazol-*1H*-yl)-acetamide
K84		N-methyl-2-(4,5,6,7-tetrabromo-benzimidazol-*1H*-yl)-acetamide
K85		(4,5,6,7-tetrabromo-benzimidazol-*1H*-yl)-acetic acid hydrazide

Table9: Tetrabromo-imidazole derivatives

Table 10 shows the inhibitory potency of all new derivatives toward protein kinase CK2. Whenever applicable, K_i values of new iodinated inhibitors are reported in comparison with the analogous brominated compounds either newly synthesized and tested here or drawn from the literature.

A Iodinated compound	K_i (μM)	B Brominated compounds	K_i (μM)	Ratio B/A
K88 (TIBI)	0.023	K17 (TBI)	0.30	13.04
K105	0.16	-	-	-
K104	0.10	-	-	-
K98	0.46	-	-	-
K106	0.59	-	-	-
K95	0.075	-	-	-
K92	0.024	-	-	-
K99	0.33	-	-	-
K93	0.019	-	-	-
K89	0.12	K10	0.37	3.08
K94	0.07	K11	0.20	2.87
K103	0.12	K21	0.25	2.08
K96	0.09	K20	0.37	4.11
K100	0.027	K30	0.14	5.18
K97	0.05	K22	0.20	4.00
K101	0.054	K33	0.12	2.22
K112	0.05	K32	0.18	4.00
K107	0.14	K68	1.34	9.57
K108	0.13	K83	1.45	11.15
K109	0.18	K84	2.31	10.60
K110	0.16	K85	1.51	9.40

Table10: Inhibition of protein kinase CK2 by iodinated benzimidazole derivatives (A) in comparison to respective brominated compounds (B)

As a main outcome of these determinations we observed firstly that the iodinated inhibitors behave in general more efficiently than the corresponding brominated ones with K_i values for CK2 which, in some cases, reach the low nanomolar range. In particular, the inhibition constants of tetraiodinated compounds K88, K92, K93, and K100 (23, 24, 19, and 27 nM, respectively) are among the lowest values ever reported

in literature for CK2 inhibitors. Also these compounds, as it was previously found with similar polyhalogenated derivatives[122], behave as competitive inhibitors with respect to the phosphodonor nucleotide, displaying a classical double-reciprocal plot allowing to calculate, in the case of 4,5,6,7-tetraiodobenzimidazole (K88 or TIBI), a K_i value of 23 nM (figure: 27A).

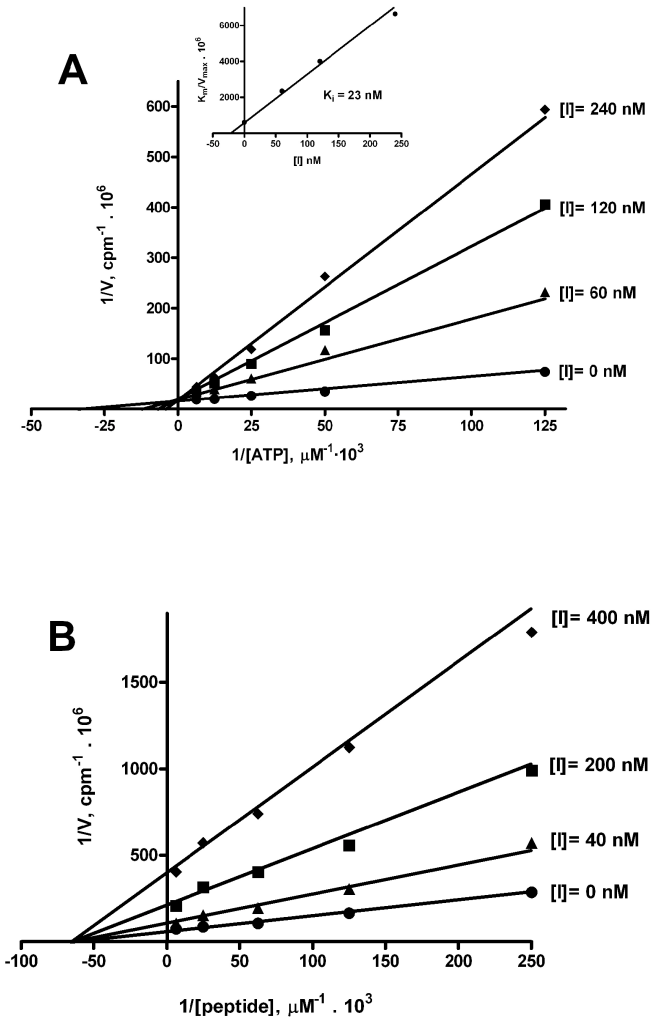


Figure. 27: Double-reciprocal plot analysis of CK2 inhibition by compound K88 (TIBI). The Ki of the inhibitor competitive with respect to ATP was then calculated by linear regression analysis of K_m/V_{max} versus inhibitor concentration plot.

Conversely, inhibition is non-competitive with respect to the peptide phosphoacceptor substrate (figure: 27B) further supporting a mechanism of action implying the occupancy of the nucleotide binding pocket in competition with ATP. Moreover, the performance of compound K88 allows to definitely rank the efficiency of halogens as substituents of the benzene ring, iodine being the best, followed by bromine (0.023 vs

0.3 μM by comparing, compounds K88 and K17 (also termed TBI) which in turn proved to be about two orders of magnitude more effective than chlorine, 4,5,6,7-tetrachlorobenzimidazole (TCI) exhibiting a K_i value of 21 μM under comparable conditions[176]. This did not come as a surprise considering the data already available for di-substituted benzimidazole derivatives in which fluorine was found to be the least effective[92,105].

However, a deeper analysis of the K_i values of tetraiodinated versus tetrabrominated compounds highlights that the presence of an additional substitution on the imidazole ring may affect not only the absolute potency of the inhibitor but also its relative efficacy with respect to the brominated homologue. In fact, all the four compounds bearing a substitution at position 1 of the imidazole ring (compounds K107, K108, K109 and K110 of table 8) display a K_i value about one order of magnitude higher than that of derivative K88 having no substitution at that position. Nevertheless, they are still 10-fold more potent than the correspondent brominated derivatives as it is observed in the case of the un-substituted compound K88. On the contrary, the added value of the presence of four iodine atoms on the benzene moiety appears to be variably affected by substitutions at position 2 of the imidazole ring. These latter compounds, with very few exceptions, display inhibition constants significantly higher and close to those of the corresponding brominated derivatives.

Searching for a plausible structural explanation of the superiority of tetraiodinated derivatives as CK2 inhibitors, a molecular modeling approach, performed by Dr.Giorgio Cozza (University of Padua) was undertaken. Starting from the crystal structure of K17 in complex with *Zm*CK2α subunit (PDB code 2OXY)[171] we determined the binding mode of compound K88 by a molecular docking strategy, in comparison with that of the tetrabromo (TBI, K17) and tetrachloro (TCI, K113) homologues, using human CK2 as target protein (PDB code: 1JWH) [103] (figure 28).

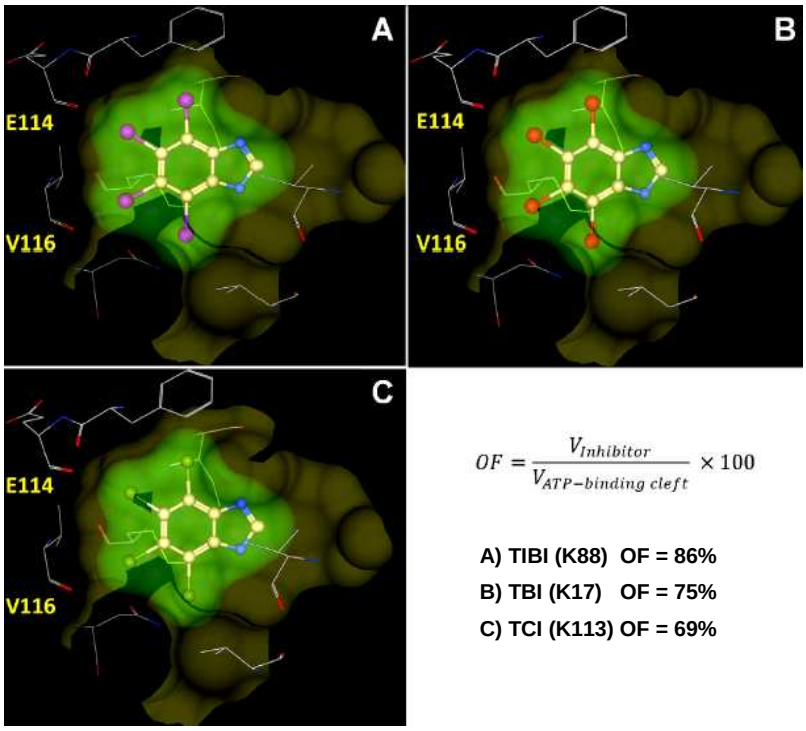


Figure 28: Molecular docking of the tetraiodinated (A), tetrabrominated (B) and tetrachlorinated (C) benzimidazole derivatives bound to the active site of protein kinase CK2. Connolly's distribution surfaces of the three inhibitors inside the nucleotide pocket are represented in light green. The occupancy factor (OF) values, discussed in the text, are also indicated.

All three inhibitors lay inside the CK2 binding cleft exactly at the same position as K17 in *Zm*CK2α, revealing identical binding modes. However, compound K88 is able to fill better the CK2 binding cleft due to the increased dimensions of iodine substituents with respect to bromine and chlorine. To better validate this hypothesis we have calculated the percentage of human CK2 ATP-binding cleft occupancy (occupancy factor, OF) by the three analogs. The CK2 ATP-binding pocket volume was calculated by using the human CK2α crystal structure from PDB (code 1JWH)[103] and processed in order to remove the ligands and all water molecules except to the one conserved[171]. Hydrogen atoms were added to the protein structure using standard geometries with the MOE program. To strictly validate the model generated and to calibrate the high-throughput docking protocol, a small database of known CK2 inhibitors was built and a set of docking runs was performed. The OF values were calculated relative to the binding cleft and the inhibitor's volume. As also shown in figure 28, TCI shows a low binding cleft OF value (69%), and tetrabromine substitution is responsible of an increased occupancy up to 75%, while the most potent inhibitor K88 (TIBI), bearing the iodine

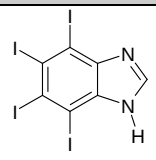
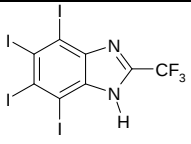
substitution, fills by 86% the CK2 ATP binding pocket. Almost identical conclusions can be drawn by comparing the increased hydrophobicity with the increased inhibitory potency of the tetrahalogenated derivatives.

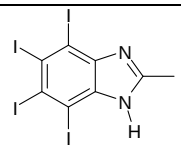
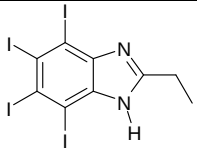
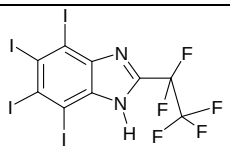
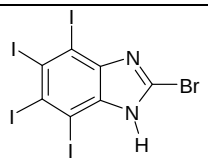
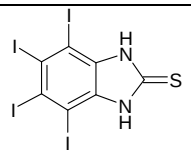
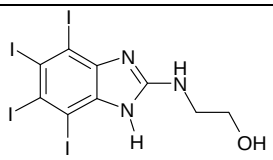
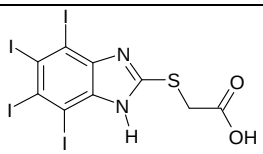
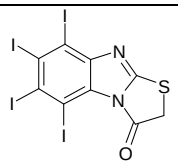
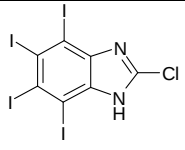
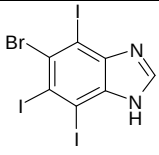
Moreover, iodine atoms of derivative K88 are able to perform stronger halogen bonds with the binding cleft, in particular with the backbone carbonyls of Glu114 and Val116 of the hinge region[171]. In fact, even if all four halogen atoms are capable of acting as halogen bond donors, iodine usually forms the strongest interactions, followed by bromine and chlorine atoms, respectively[177]. The same arguments apply also to the other tetraiodo-benzimidazole derivatives considered in this work, all displaying in general an inhibitory efficiency higher than that of their brominated counterparts.

5.2.2 SELECTIVITY OF TETRAIODOBENZIMIDAZOLE DERIVATIVES

To study the selectivity of tetraiodobenzimidazole derivatives we tested some of these compounds on an “in-house” panel of protein kinases.

In particular we have focused our attention on two kinases which usually are affected by the inhibitors of CK2, such as PIM1 and HIPK2. In fact, as we have demonstrated in our laboratory, some important benzimidazole inhibitors of CK2, such as TBB and its analogue K17 (TBI) appeared to be good inhibitors also of other kinases, in particular of the two kinases mentioned above[178]. In table 11 the different activities of tetraiodobenzimidazole derivatives on these three kinases have been reported.

Compound	**Structure**	IC_{50} on **nCK2** at 20μM [ATP]	IC_{50} on **PIM1** at 100 μM [ATP]	IC_{50} on **HIPK2** at 20μM [ATP]
K88		0.038	0.32	0.87
K89		0.229	1.63	3.65

K92		0.046	0.35	0.31
K93		0.039	0.28	0.31
K94		0.15	1.81	2.15
K96		0.19	0.88	0.61
K97		0.10	0.28	0.34
K100		0.054	0.26	0.28
K101		0.108	0.72	0.77
K102		0.031	0.46	0.23
K103		0.24	0.89	1.67
K104		0.21	0.46	0.32

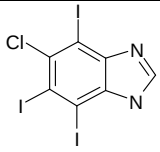
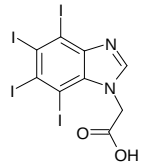
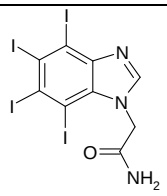
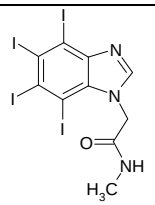
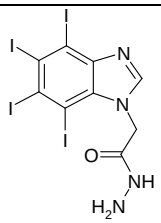
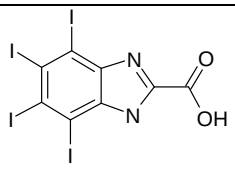
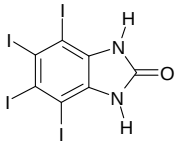
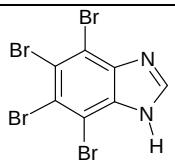
K105		0.32	0.42	0.31
K107		0.28	4.58	0.93
K108		0.27	0.50	1.68
K109		0.36	1.41	12.9
K110		0.32	0.68	40.0
K111		0.014	0.31	8.8
K112		0.084	0.34	4.28
K113		16.5	3.03	33.45

Table11: IC_{50} of poly-iodinated derivatives for CK2, PIM1 and HIPK2.

To note that most compounds (14 respect to a total of 20) are able to inhibit also PIM1 and HIPK2 with values of IC_{50} comparable to those displayed by CK2 in the low micromolar or close to micromolar range.

Normally a substitution at position 2 reflects a loss of activity on CK2 while the inhibitory potency on PIM1 and HIPK2 is apparently not altered. On the other hand the insertion of a carboxylic group at the same position (in the case of K111) increases the activity towards CK2 (IC_{50} = 0.014μM) by decreasing at the same time the potency against HIPK2. So this latter compound is more selective for CK2.

From this point of view also the substitutions at position 1 are promising. In fact the insertion of a negative charge (for example that produced by a $–CH_2COOH$ group) makes K107 a poor inhibitor of PIM1 confirming some recent results [178], suggesting that PIM1 does not tolerate a highly hydrophilic group in this position.

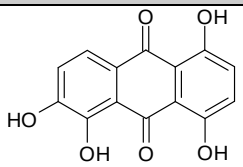
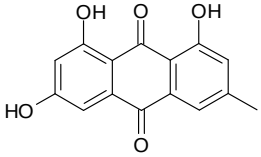
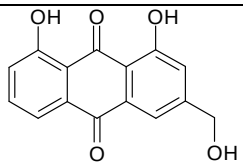
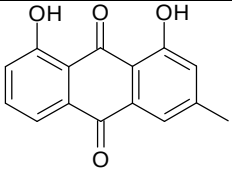
To confirm this, the substitution of the carboxylic group with the corresponding amide (K108 and K109) or hydrazide (K110) groups lowers the inhibition to the level of control (K88).

Conversely, the elongation of the chain at position 1 results in a loss of inhibitory activity against HIPK2, (for example in the case of K110), which becomes almost 46-fold less potent on this kinase with respect to K88. On the contrary, CK2 and PIM1 are only slightly affected by this substitution, by showing only 8- and 1.6-fold higher IC_{50} values, respectively. It is also important to underline that the substitution of only one iodine atom with a chlorine (K105) and even more the complete subtitution of all atoms (K113) is well-tolerated by PIM1 which, at most, reduces its IC_{50} 9.4-fold.

5.3 QUINALIZARIN[179]

5.3.1 GENERAL

The anthraquinone emodin was one of the first scaffold to be identified as a potential inhibitor of CK2[171] with a K_i value of about 1 μM. Emodin has represented the starting point for a series of new anthraquinone derivatives in which, besides some hydroxyl group, other electron-attractor groups were added to increase the inhibitory activity.
This idea was firstly confirmed by the synthesis and biochemical evaluation of some anthraquinones with a structure similar to emodin such as 1,8-dihydroxyanthraquinone, chrysophanic acid, aloe-emodin and 1,4,5,8-tetrahydroxyanthraquinone, which proved to be completely inactive (shown in table12).
On the contrary, MNA (1,8-dihydroxy-4-nitro-anthraquinone) resulted to be a rather effective inhibitor of CK2[118].

Inhibitor	Structure	Name	IC_{50} (μM)
Quinalizarin		1,2,5,8-tetrahydroxy-anthraquinone	0.11
Emodin		1,3,8-trihydroxy-6-methylanthraquinone	1.90
Aloe emodin		1,8-dihydroxy-3-hydroxymethyl-anthraquinone	28.0
Chrysophanic acid		1,8-dihydroxy -3-methyl-anthraquinone	>40.0

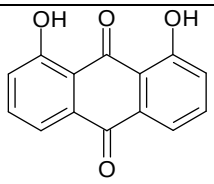
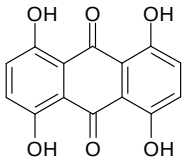
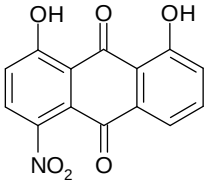
A1		1,8-dihydroxy-anthraquinone	>40
A2		1,4,5,8-tetrahydroxy-anthraquinone	>40
MNA		1,8-dihydroxy-4-nitro-anthraquinone	0.30

Table12: IC_{50} of quinalizarin and other anthraquinones for holoenzyme CK2

5.3.2 ACTIVITY OF QUINALIZARIN

More recently, in a computer-aided virtual screening of the MMS database, performed by Dr giorgio Cozza, based on the crystal structure of human CK2 (PDB code 1JWH), quinalizarin (table12) has been found to sit in the top 10% of the ranked database independently of the nature of the scoring function used. This prompted us to assay quinalizarin as a CK2 inhibitor, by performing the kinetic experiments illustrated in figure 29

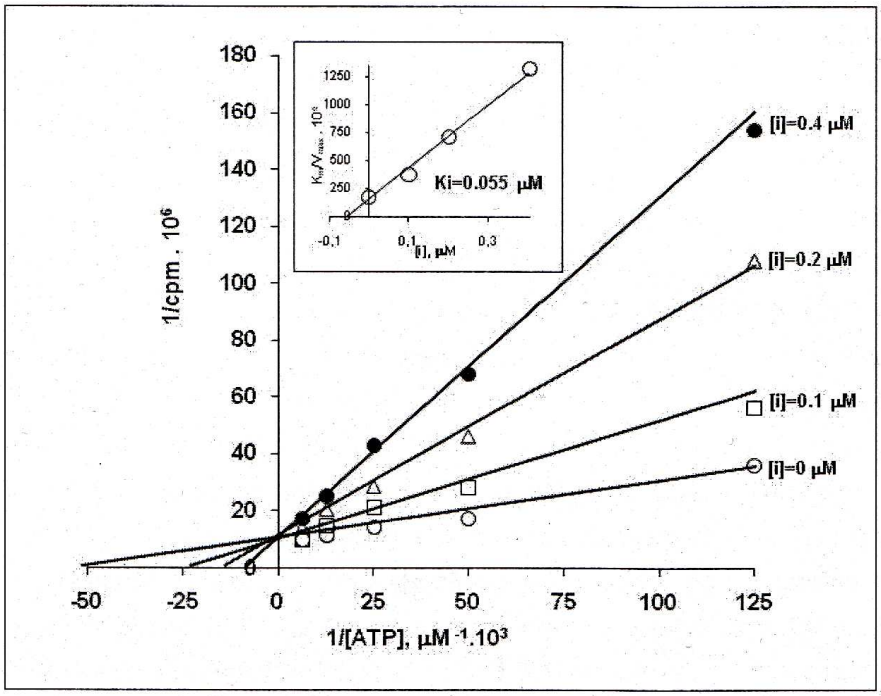


Figure 29: Kinetic analysis of CK2 inhibition by quinalizarin

We have performed an experiment at increasing concentration of ATP, in absence and in presence of some fixed concentrations of quinalizarin (0.1, 0.2 e 0.4 µM) in order to calculate the K_i value.

The results show that quinalizarin is indeed a powerful inhibitor of CK2, competitive with respect to ATP: its K_i value (approx. 60 nM) is lower than those of emodin and TBB[121] and comparable with that of TBCA [120].

5.3.3 SELECTIVITY

Next, we have studied the selectivity of quinalizarin by testing the inhibitory potency on a panel of 75 protein kinases. The selectivity profile was run at a concentration of 1 µM, sufficient to inhibit CK2 activity more than 90%. As shown in figure 30, under these conditions, the residual CK2 activity was approx. 7%. In sharp contrast, none of the other kinases was inhibited as drastically as CK2, with the second most inhibited kinase (PIM3) still exhibiting more than 50% residual activity, followed by DYRK1a, DYRK3 and PIM1, whose residual activity was 60% or more.

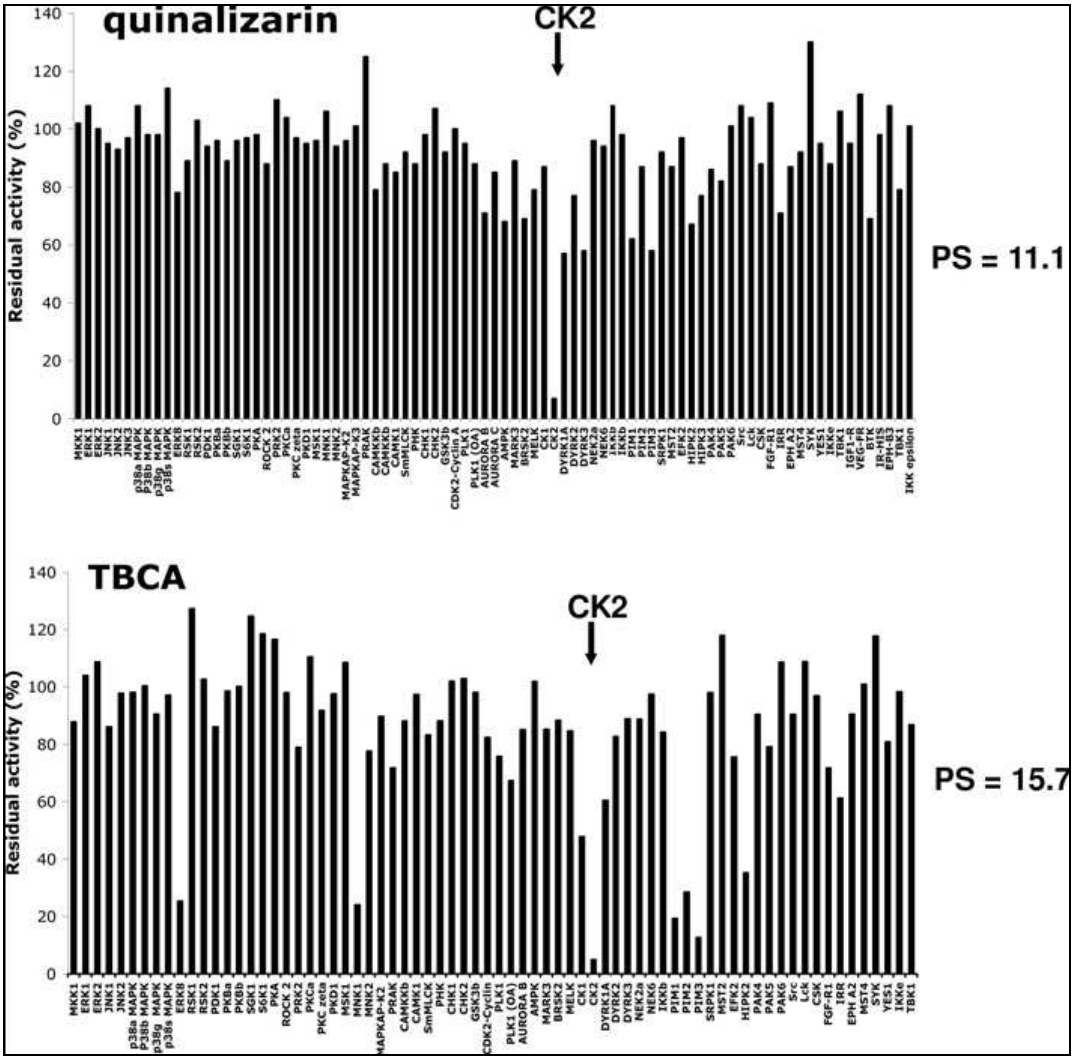


Figure 30: Selectivity profiling of quinalizarin and TBCA. Inhibition assays were performed at a concentration of 1 µM of the indicated inhibitor. On the right, the values of promiscuity scores (PS) are shown.

The K_i values of quinalizarin against these kinases were calculated (table 13). It was possible to note that quinalizarin shows K_i values one or two orders of magnitude higher with respect to CK2, a difference sufficient to ensure a clear-cut discrimination of CK2 among other kinases susceptible to quinalizarin inhibition. In contrast, some of these kinases, notably PIM1 and PIM3, were inhibited by emodin as well as by the commercially available CK2 inhibitors TBB, DMAT and TBI/TBBz more drastically than by quinalizarin.

Inhibitor	K_i (μM) on:				
	CK2	DYRK1a	PIM1	PIM3	HIPK2
Quinalizarin	0.052	5.850	1.392	1.205	1.956
Emodin	1.250	2.815	0.283	0.048	8.105
TBB	0.049	2.906	0.690	0.320	3.096
TBI	0.139	1.408	0.076	0.026	0.550
DMAT	0.045	0.270	0.096	0.036	0.212

Table13: K_i of quinalizarin and of other known CK2 inhibitors on CK2, DYRK1a, PIM1, PIM3 and HIPK2.

The remarkable selectivity of quinalizarin prompted us to compare it with that of TBCA, another CK2 inhibitor whose efficacy is sufficiently high to profile its selectivity at a concentration of 1 instead of 10 μM. TBCA was tested previously at a concentration of 10 μM on a panel of 28 protein kinases, disclosing its ability to discriminate between CK2 and DYRK1a[120], a kinase often affected by CK2 inhibitors. That panel, however, did not include a number of kinases shown later to be inhibited by TBB, DMAT and other CK2 inhibitors as drastically as CK2 itself [178]. The new data with TBCA (1 μM) on a panel of 72 protein kinases (performed in the laboratory of Professor Sir Philip Cohen, Dundee, Scotland) are shown in figure 30 (lower panel), where they are compared with those obtained with 1 μM quinalizarin (upper panel). They confirm that kinases of the DYRK family are much less susceptible to TBCA (as well as to quinalizarin) than is CK2. However, they also show that kinases of the PIM family tend to be inhibited more drastically by TBCA than they are by quinalizarin and that two other kinases either unaffected or poorly affected by quinalizarin, MNK1 [MAPK (mitogen-activated protein kinase)-interacting kinase 1] and ERK8, are inhibited 75% or more by 1 μM TBCA.

Higher selectivity towards CK2 of quinalizarin compared with TBCA is also reflected in a lower promiscuity score, expressing the average inhibition of the protein kinases of the panel by an inhibitor concentration sufficient to suppress CK2 activity (residual activity <10%)[179]. As calculated in figure 30, the promiscuity score of quinalizarin

(11.1) is below that of TBCA (15.7). It is also much lower than those calculated for the typical CK2 inhibitors TBB, TBI/TBBz and DMAT[178].

In summary, it appears that quinalizarin is the most selective CK2 inhibitor analysed, with a potency, in terms of a K_i value, comparable with those of the most powerful of these inhibitors, notably TBCA, DMAT, DBC and NBC[118,120]. Somewhat surprisingly, the two unique CK2 hydrophobic residues shown to play a prominent role in the interaction with the majority of CK2 inhibitors, Val66 and Ile174, appear to be only of marginal relevance in the case of quinalizarin, whose IC_{50} value is poorly affected by their mutation to alanine. In contrast, these mutations cause a more than 50-fold increase in the IC_{50} for emodin (figure 31).

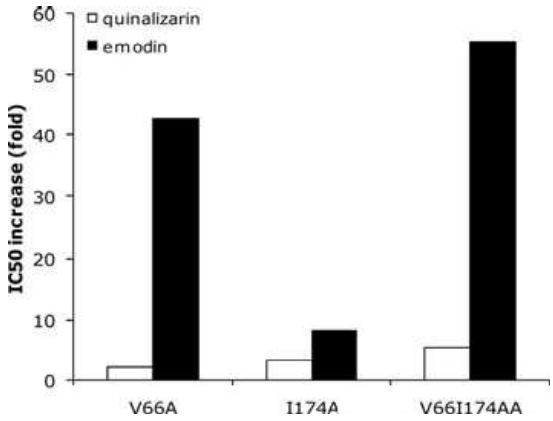


Figure 31: CK2 inhibition by quinalizarin is marginally affected by Val66 and Ile174 substitutions

To get a better insight into the mode of binding of quinalizarin and to try to disclose the structural basis for its selectivity, the three-dimensional structure of a complex between quinalizarin and CK2α (from *Z. mays*) has been solved (performed by Professor Roberto Battistutta, Padova, Italy). As shown in figure 32(A), quinalizarin makes polar interactions with CK2 using at least three of its hydroxy groups: the one at position 2 is

hydrogen-bonded with the conserved water molecule (1) and Lys68 (which is also near to quinalizarin hydroxy group 2, 3.3 Å from it), the one at position 5 interacts with the hinge region (carbonyl backbone of Val116) through another water molecule, whereas hydroxy group 8 makes a very stable polar interaction with His160 and the backbone carbonyl group of Arg47.

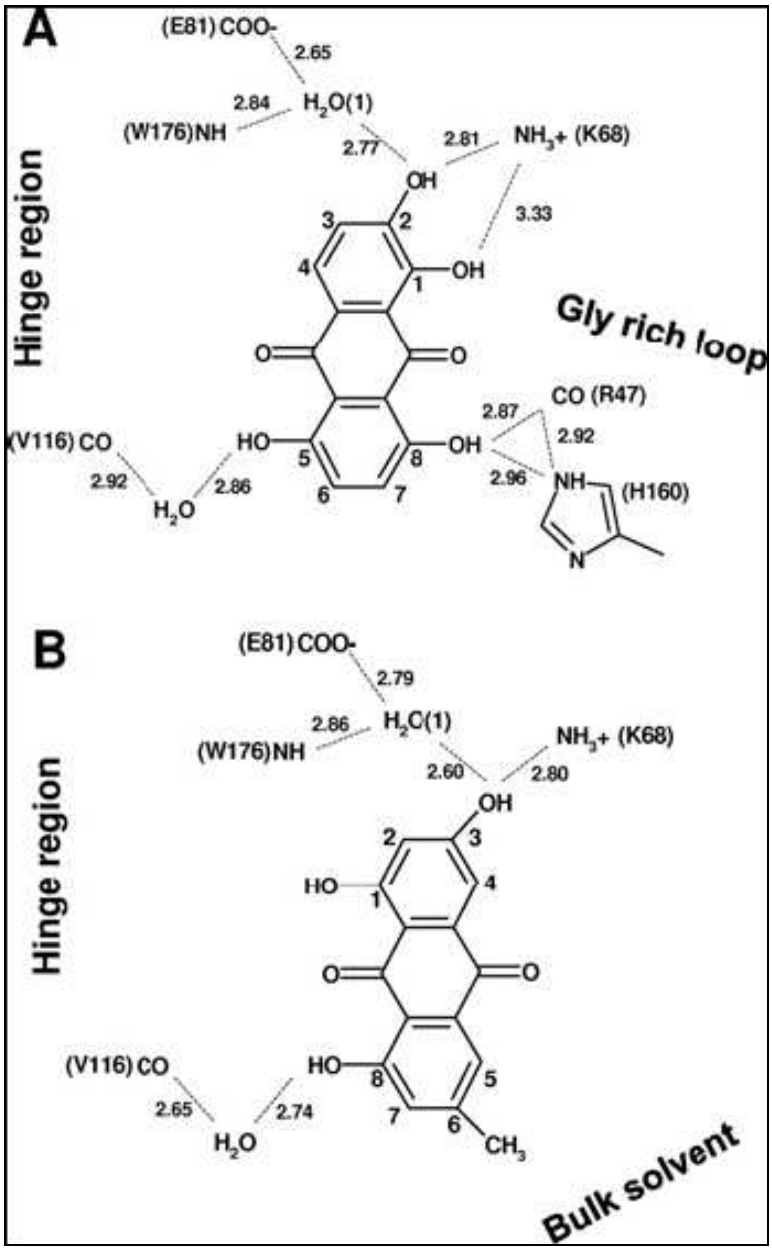


Figure 32 Structural insight into the CK2*α*–quinalizarin complex

The two-dimensional cartoon summarizes the main polar interactions observed in the crystal structures of CK2*α* complexed with quinalizarin (A) and emodin (B).

This latter interaction occurs through hydrogen bonds among the three atoms at almost the same distance, giving rise to a sort of equilateral triangle and stabilizing the kinase into a close conformation which entraps the inhibitor inside the pocket (figure 33).

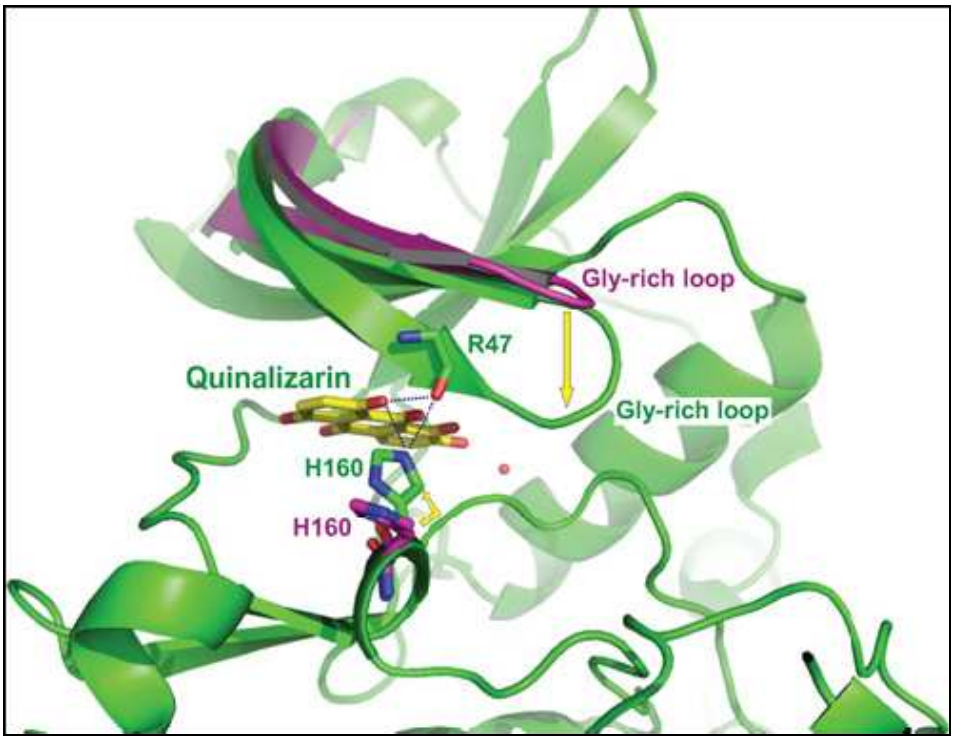


Figure 33 Close-up view of the inhibitor quinalizarin (yellow carbon atoms) bound to CK2 (in green)

Note in this respect that, although the scaffold of quinalizarin in this structure is coplanar and superimposable on that of emodin in its complex with human CK2 (PDB code 3BQC, see figure 32B)[181] the lack of a hydroxy group at position 5 in emodin prevents the formation of the interaction with His160 and the backbone of Arg47 found with quinalizarin.

Since crystallographic data are not yet available for PIM1 kinase in complex with quinalizarin or emodin, a molecular docking of these inhibitors with PIM1 was performed (by dottor Giorgio Cozza, Padova) (figure 34).

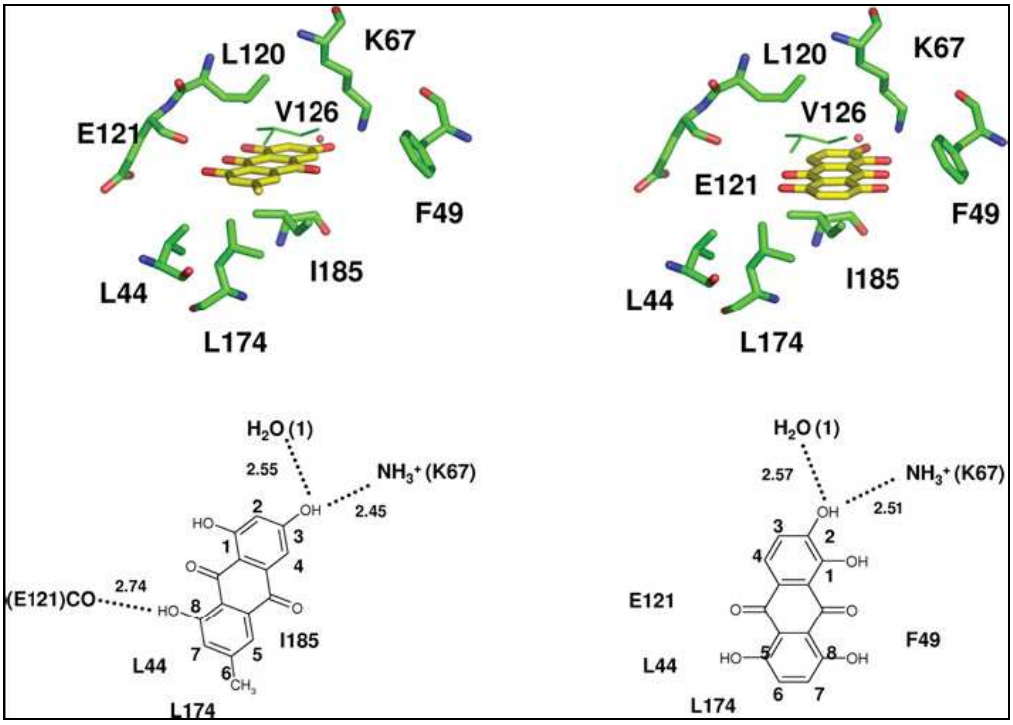


Figure 34: Molecular docking of quinalizarin by PIM1

This discloses structural features accounting, on the one hand, for the higher susceptibility of this kinase to emodin, and on the other, for its reduced sensitivity to quinalizarin. Although both inhibitors can be accommodated in the binding cleft of PIM1 in a position similar to that observed in the two crystal structures of CK2 discussed above (PDB codes 3BQC and 3FL5 for emodin and quinalizarin respectively), they have to face at least three important amino acid substitutions.

First, the replacement in PIM1 of the CK2 water-exposed Tyr50 with Phe49 (PIM3 carries the same substitution), which is oriented inside the cleft, puts both inhibitors in contact with this hydrophobic side chain; whereas emodin can take advantage of this apolar interaction owing to its more hydrophobic characteristics, Phe49 will negatively affect quinalizarin binding, mainly for an unfavourable interaction with the hydroxy group at position 8, at only 3.6 Å from the phenylalanine aromatic side chain. Note that in the corresponding position (5) emodin does not carry any functional group.

Secondly, PIM1 replaces CK2 Met163 with Leu174. Also in this case, the docking mechanism suggests an unfavourable interaction between the hydroxy group at position 5 and Leu174, at only 3.5 Å. In this situation, the hydroxy group at position 5 cannot make an interaction with a water molecule as in the case of CK2. On the other

hand, emodin is attracted towards Leu174, preserving the interaction between the hydroxy group at position 3 and the conserved water molecule, but making a rotation of approx. 30° with respect to the quinalizarin position. This places emodin in a more favourable hydrophobic subsite, formed by Leu44, Val126 and Leu174.

At the same time, with this new interaction, the hydroxy group at position 8 of emodin is hydrogen-bonded to the backbone carbonyl group of Glu121, at 2.7 Å distance. Finally, owing to the substitution of Glu171 for His160, PIM1 is not able to make the same interaction of quinalizarin's hydroxy group at position 8 observable in the crystal structure of its complex with CK2.

It is interesting to note that only 21% of the protein kinases included in the selectivity panel have a tyrosine residue at the position homologous with CK2 Tyr50 and only 10% have a methionine residue homologous with CK2 Met163. Almost all the kinases belonging to this latter group have a phenylalanine residue instead of Tyr50. Moreover, within the selectivity panel, CK2 is the only kinase bearing the His160 residue. It can be concluded therefore that CK2 possesses a particular combination of residues that could explain the remarkable selectivity of quinalizarin.

5.4 1,4-DIAMINOANTHRAQUINONE

This part of the work is dealing with the studies of the ability of some compounds active on protein kinase CK1 to discriminate among its different main isoforms.

Protein kinase CK1 represents a unique and well conserved group of protein kinases within the superfamily of serine/threonine kinases that is ubiquitously expressed in eukaryotic organisms. Recently, seven mammalian CK1 isoforms have been identified (α, β, γ1, γ 2, γ 3, δ, ε) with a molecular weight between 37 kD and 51 kD. Even if all CK1 isoforms are highly conserved within their kinase domains, they show important differences in length and primary structure of the N-terminal and C-terminal domains[182]. As already discussed in the introduction, CK1 isoforms appear to be costitutively active with a consensus motif pS-X-X-S.

In recent years we have performed an intensive screening campaign, using an integrated *in silico* approach, and verifing computational results with a biochemistry approach[183]. In particular we have focused our attention on the CK1δ isoform due to its key role in both neurodegenerative diseases and cancer.

Following some recent successful examples of new kinase inhibitors discovery by high-throughput docking (HTD), Dr Giorgio Cozza performed a virtual screening targeting the ATP binding site of CK1 by browsing our “in house” molecular database (defined as MMsINC) which count around 4 of millions synthetic and natural compounds[184].
Specifically, a combination of three docking protocols (MOE-Dock, Glide, and Gold)[184] and five scoring functions (MOE-Score, GlideScore, Gold-Score, ChemScore and Xscore)[184] has been used to appropriately dock and rank all MMsINC candidates. Due to the fact that no crystal structure is available for the human CK1δ an homology modeling approach has been carried out to obtain a suitable model of the CK1δ catalytic subunit.
MMsINC database. MMsINC® is a free web-oriented database of commercially available compounds for virtual screening and chemoinformatic applications**.** MMsINC contains over 4 million *non-redundant* chemical compounds in 3D format. The whole database was studied in term of uniqueness, diversity, frameworks, chemical reactivity, drug-like and lead-like properties. This study shows that there are more than 175.000

frameworks in our database. There are 3.89 millions (98%) of drug-like molecules among which more than 3.61 millions (91%) are lead-like. Moreover, 3.45 millions (87%) are considered chemically stable compounds. The druglikeness and leadlikeness are estimated using Lipinski and Oprea cut-off values. The compounds are stored in a PostgreSQL database and the code to manage this database is in Java. Moreover, MMsINC® is nicely integrated with PubChem and PDB databases facilitating the cross exchange of ligand information. In consequence, we have a free and easily updatable system for chemical databases management and screening sets generation. MMsINC® is accessible at the following web address: http://mms.dsfarm.unipd.it/MMsINC/.

Interestingly, a small family of anthraquinone derivatives has been found to sit on the top 10% of the ranked database, independently from the nature of the used scoring function. In particular the compound **1**, shown in Table 14, was one of the best ranked compounds even better then all known CK1 inhibitors. Considering the encouraging virtual screening results, we have prioritized the acquisition and the biochemical characterization of derivative **1** as new potential CK1δ inhibitor.

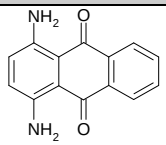
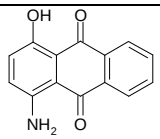
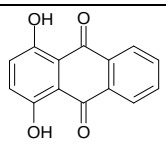
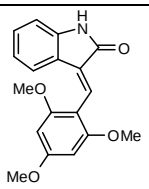
Name		CK1δ	CK1γ1	CK1α
1 1,4-diamino-anthraquinone		0.33	34	4
2 1-hydroxy-4-amino-anthraquinone		0.66	26.2	4
3 1,4-dihydroxy-anthraquinone		>40	>40	>40
IC261		2.57	>40	1.24

Table14: Inhibition of CK1 isoforms by CN calculated as IC_{50} (µM)

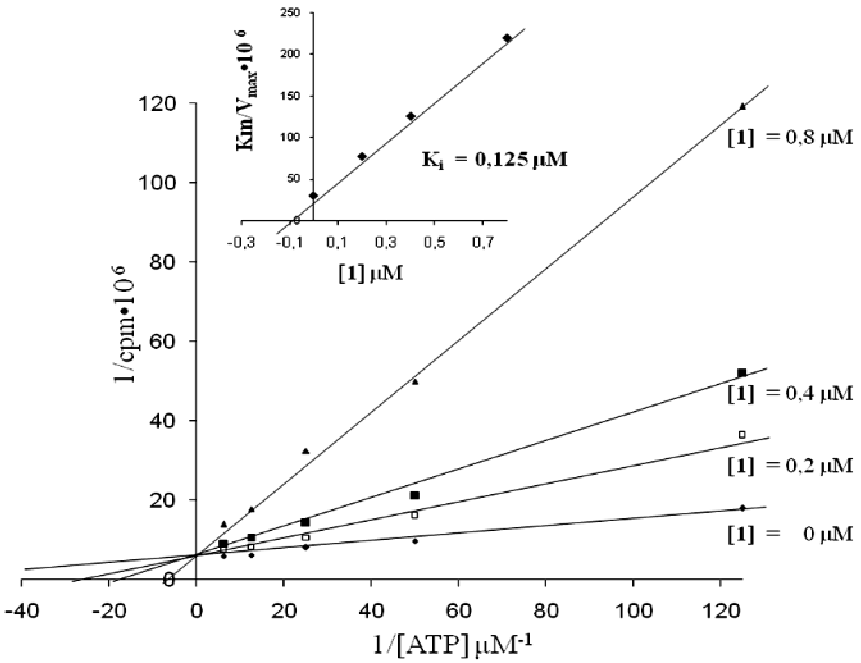


Figure 35: Kinetic analysis of compound **1** on CK1δ complexation consistent with a reversible and competitive mechanism of inhibition.

As shown in figure 35, inhibition of CK1δ by compound **1** is competitive with respect to the phosphodonor substrate ATP, and a 125 nM K_i value has been calculated from linear regression analysis of Lineweaver-Burk double reciprocal plots, which is the one of the lowest K_i reported so far of any CK1δ inhibitor.

CK2	HIPK2	PIM1	PKA	CSK	LYN	SYK	FGR	GST-ALK
18	3.3	24.7	>40	>40	>40	>40	24	>40

Table15. Inhibition of selected protein Kinases by compound **1** calculated as IC_{50} (µM)

On the other hand, according to a preliminary selectivity study (table 14 and table 15), derivative **1** seems to be a quite specific inhibitor of CK1δ with respect the other CK1 isoforms, and also with respect a small panel of different kinases.

From a biochemical point of view, similar to other CK1δ inhibitors such as IC261 (shown in table 14) and others, derivative **1** is an ATP-competitive inhibitor, and, as

showed in figure 36 our molecular docking investigation, have clearly shown that derivative **1** displays a very good steric and chemical complementarity with the ATP binding cleft. Derivative **1** lies essentially in the same plane of all other known crystalographic inhibitors, however presents a particular binding motif, interacting both with the hinge region and the phosphate-binding region of the ATP-binding cavity.

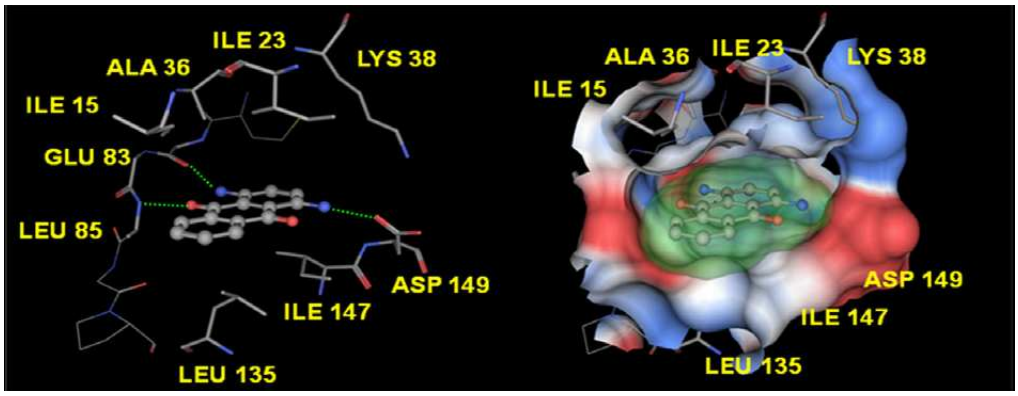


Figure 36: Molecular docking of compound 1 bound to the active site of the CK1δ catalytic subunit. On the left, analysis of the binding mode of derivative 1 whose interactions with the most crucial amino acids are highlighted. On the right, Connolly's electrostatic charge distribution surface of ATP-binding cleft of CK1δ (blue indicates positive surface charge and red indicates negative surface charge).

In fact, in this binding configuration, derivative **1** makes a stabilizing interaction between the amino group at the 1-position and the backbone carbonyl of Glu83 in the hinge region; moreover the carbonyl group near 1-position can make another good interaction with the backbone amino group of Leu85. Notably, these are the same interactions involved in the binding of adenine moiety of ATP to the active site. On the other hand, another hydrogen-bonding interaction has been detected between the amino group 4-position and the carboxylic group of Asp149. Moreover, we can't forget several hydrophobic interactions (Ile15, Ile23, Ala36, Leu135, Ile147) which contribute to strongly stabilize compound in complex with CK1δ. In conclusion the right balance of both polar and hydrophobic interactions and the perfect shape complementarity with the ATP-binding cleft are ultimately responsible for the high potency of derivative **1**.

Beside derivative **1**, other interesting CK1δ inhibitor candidates have been selected from our consensus ranking list and submitted to a biochemical characterization. In

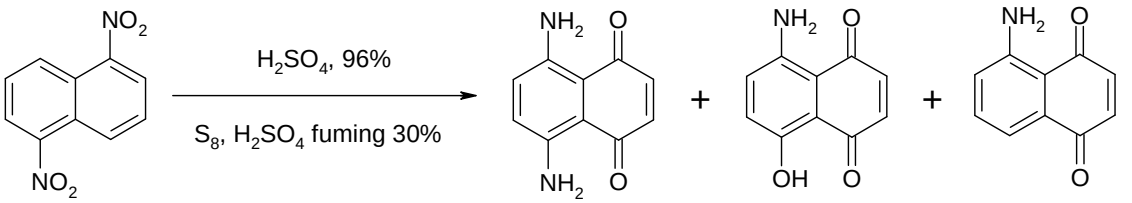
particular, the compound **2** also shows an appreciable inhibitory activity against CK1δ (IC_{50} = 0.6 μM) (table 14). To better define the role of the 1,4-diamino substituents, we have also analyzed the corresponding 1,4-dihydroxy-anthraquinone as potential CK1δ inhibitor. Interestingly, this compound shows drastically lower inhibitory effect (IC_{50} > 40 μM) on δ isoform than derivative **1** and **2**.

A computer-aided modellization of the complex between CK1δ and compound 1 seems to suggest the importance of the two amino groups at positions 1-4 which are in contact with the carboxylic group of Asp149 and the backbone carbonyl of Glu83 in the hinge region respectively.
Moreover, one of the carbonyl groups can interact through an H bonding with the backbone amido moiety of Leu85. These two interactions are those generally observed in the binding of adenine moiety of ATP into the kinase active site. Finally, several hydrophobic interactions (Ile15, Ile23, Ala36, Leu135, Ile147) may contribute to stabilize the complex between compound 1 and CK1δ. Probably the right balance of both polar and hydrophobic interactions and the appropriate shape complementarity with the CK1δ ATP-binding cleft might be ultimately responsible for the appreciable inhibitory activity and for the selectivity of DAA versus other kinases and in particular against the protein kinase CK2.

For studying better the SAR of DAA we have decided to test some compounds displaying simplyfied structure (benzene and naftoquinone derivatives). Some of them are commercially available (compounds 1-7), while others have been synthesized in our laboratory (compounds 27-29).

5.4.1 Chemistry

Synthesis of 5,8-diamino-[1,4]naphthoquinone (27), 5-Amino-8-hydroxy-[1,4]naphthoquinone (28) and 5-Amino-[1,4]naphthoquinone (29)



27 $C_{10}H_8N_2O_2$ MW =188,1875

28 $C_{10}H_7NO_3$ MW =189,1722

29 $C_{10}H_7NO_2$ MW =173,1728

To an ice-cooled slurry of 1,5-dinitronaphthalene (2g, 9.2 mmol) in concentrated sulphoric acid (4.5 ml) a mixture of sulphur (750 mg) and fuming sulphoric acid (30% SO_3, 8.5 ml) is added dropwise with stirring.

Stirring is continued for one hour and then the mixture is warmed at 50℃ for 10 minutes.

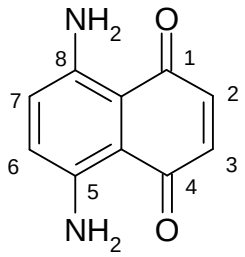
The mixture is cooled, allowed to stand at room temperature for 18 hours, and finally poured on crushed ice.

The solution is filtered and extracted with chloroform in a liquid/liquid extractor.

By TLC ($CHCl_3$:MeOH/9:1) we can see the presence of 3 products, which are separated by column chromatography on silica gel ($CHCl_3$:MeOH/9:1).

The compounds have three different colors: the first eluting from the column is the compound 29 and appears red (319 mg, yield 20%), the second is violet (435 mg, yield 25%) and corresponds to the compound 28 and the last is the compound 27 which is blue (587 mg, yield 34%).

5,8-diamino-[1,4]naphthoquinone (27)

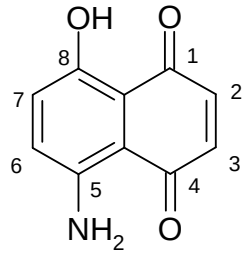


^{1}H NMR [300 MHz, CD_3OD] δ 7.21 (s, 2H, **H2**, **H3**), 6.90 (s, 2H, **H6**, **H7**)

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 182.22; 182.22; 141.34; 141.34; 138.96; 138.96; 123.43; 123.43; 112.98; 112.98;

HRMS calcd for $C_{10}H_8N_2O_2$ $[M + H]^+$, 189.0659; found, 189.0678.

5-Amino-8-hydroxy-[1,4]naphthoquinone (28)

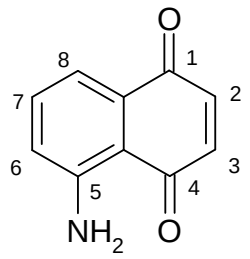


^{1}H NMR [300 MHz, CD_3OD] δ 7.24 (d, *J* = 9.5 Hz, 1H, **H7**), 7.15 (d, *J* = 9.5 Hz, 1H, **H6**), 6.98 (d, *J* = 10.2 Hz, 1H, **H2**), 6.93 (d, *J* = 10.2 Hz, 1H, **H3**),

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 182.24; 182.24; 152.36; 143.94; 138.99; 138.92; 124.06; 123.02; 117.09; 113.54;

HRMS calcd for $C_{10}H_7NO_3$ $[M + H]^+$, 190.0499; found, 190.0499.

5-Amino-[1,4]naphthoquinone (29)



^{1}H NMR [300 MHz, CD_3OD] δ 7.44 (dd, *J* = 8.5 Hz, *J* = 7.3 Hz, 1H, **H7**), 7.30 (dd, *J* = 7.3 Hz, *J* = 1.2 Hz, 1H, **H8**), 7.10 (dd, *J* = 8.5 Hz, *J* = 1.2 Hz, 1H, **H6**), 6.88 (d, *J* = 10.3 Hz, 1H, **H2**), 6.82 (d, *J* = 10.3 Hz, 1H, **H3**)

^{13}C NMR [300 MHz, $(CD_3)_2CO$] δ 182.21; 182.21; 151.39; 128.94; 138.84; 135.96; 132.74; 122.68; 120.44; 112.14;

HRMS calcd for $C_{10}H_7NO_2$ $[M + H]^+$, 174.0551; found, 174.0560.

5.4.2 BIOLOGICAL RESULTS

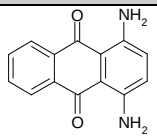
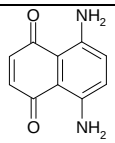
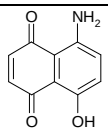
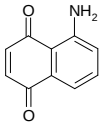
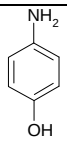
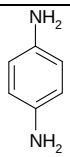
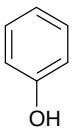
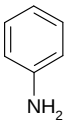
Compound	Structure	δCK1
1		**0,31**μM
27		31,6μM
28		100μM
29		54,8μM
4		>100μM
5		>100μM
6		>100μM
7		80,4μM

Table16: IC_{50} of DAA and its simplyfied structure derivatives on CK1δ at 20 μM [ATP]

From this data we can see that probably the tricyclic structure is essential for the optimal inhibition of CK1.

In conclusion, anthraquinone scaffold can be confirmed to be an interesting scaffold to design specific protein kinase inhibitors.

A deep structure-activity relationship is now running in our laboratories to clearly understand the mechanism of action of this new class of promising CK1δ inhibitors with the aim to design and synthetize novel potent and selective anthraquinone-driven CK1δ inhibitors.

Chapter 6

Conclusions

The data presented in this thesis summarize the work performed during three years of doctorate in which I shared my work between the group of Prof. F. Meggio at the Department of Biological Chemistry, where biochemical characterization of protein kinase inhibitors has been performed, and the Department of Pharmaceutical Sciences where the synthesis of the new compounds has been designed and accomplished under the supervision of Prof. G. Zagotto. Part of my work concerning the selectivity screenings of the new inhibitors was performed in Dundee during a four months stage in the laboratory of Professor Sir Phil Cohen.

The main outcomes of my studies have been described in the previous chapters of this thesis and can be summarized as follows:

1. First of all I have synthesized some simplified derivatives of Urolithin A, a metabolite found to represent the possible bioactive effector of ellagic acid, a very potent inhibitor of protein kinase CK2 recently identified in our laboratory. The data show that two new very potent inhibitors of CK2 have been identified displaying their activity in the low nanomolar range. These compounds, corresponding to compounds 20 and 24 of the Experimental Section of this thesis are derivatives of Urolithin A with a crucial substitution at position 4, given by a nitro group and a bromine in the compound 20 and 24, respectively. Bromine and nitro groups already proved in the past to often improve the efficiency of polyphenolic derivatives because they increase the acidity of para and ortho-phenolic hydroxyls within an important basic zone of nucleotide cavity of the kinase. We can say that compounds 20 and 24 can be considered among the most effective CK2 inhibitors, since the K_i value of the bromoderivative (7 nM) is one of the lowest ever detected in literature.

2. The polyiodinated benzoimidazoles have been demonstrated to behave in general much more efficient inhibitors of CK2 than the corresponding brominated ones, with K_i values reaching, in some cases, the low nanomolar range lowering by one order of magnitude the kinetic constants previously determined for polybrominated derivatives. Based on structural evidence, mainly obtained through computational molecular docking techniques, a rationale has been found for the better performance in terms of efficacy of iodine with respect to bromine and, even more, to chlorine as substituents of

the benzene moiety of these compounds, although in general these substitutions are not accompanied by a parallel increase of selectivity.

3. A remarkable result in terms of selectivity has been, on the contrary, obtained during the characterization of quinalizarin, an anthraquinone similar to emodin but displaying a much higher efficiency toward CK2. Moreover, by testing the inhibitory potency of quinalizarin on a panel of 75 protein kinases we have demonstrated that none of the other kinases tested was inhibited as drastically as CK2, with the second most inhibited kinase (PIM3) still exhibiting more than 50% residual activity. The very low K_i value of quinalizarin allowed us, in this case, to screen its activity at 1 μM a concentration sufficient to ensure a clear-cut discrimination of CK2 among other kinases possibly susceptible to quinalizarin inhibition.

4. Finally, substantial steps forward have been done in the selective inhibition of protein kinase CK1. We have actually found that DAA (1,4-diaminoanthraquinone) is the most specific inhibitor of CK1δ (IC_{50} = 0.3 μM) with negligible activity toward other CK1 isoforms, and also with respect to a small panel of other protein kinases. Also in this case structural information has been obtained by molecular docking approaches and a deep structure-activity relationship is still in progress in our laboratory to clearly understand the mechanism of action of this class of new promising CK1δ inhibitors.

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