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## COST Action CM1201 “Biomimetic Radical Chemistry”: free radical chemistry successfully meets many disciplines

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### ABSTRACT

The COST Action CM1201 “Biomimetic Radical Chemistry” has been active since December 2012 for 4 years, developing research topics organized into four working groups: WG1 – Radical Enzymes, WG2 – Models of DNA damage and consequences, WG3 – Membrane stress, signalling and defenses, and WG4 – Bio-inspired synthetic strategies. International collaborations have been established among the participating 80 research groups with brilliant interdisciplinary achievements. Free radical research with a biomimetic approach has been realized in the COST Action and are summarized in this overview by the four WG leaders.

### ARTICLE HISTORY

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Radical enzyme; membrane stress; phospholipid remodeling; DNA damage and repair; biomimetic models; bio-inspired synthetic methodologies

### Introduction

According to the Memorandum of Understanding (MoU) subscribed by the 24 participating countries, as well as by 2 COST Near Neighbor Countries and 2 International Partner Countries, the aims of the COST Action 1201 were:

- affirming the centrality of chemistry for the comprehension at a molecular level of processes in biology and technology and the use of biomimetic models as simplified, yet strictly relevant systems, for studying molecular interactions and effects;
- fostering an innovative multidisciplinary scenario ideal for Early Stage Researchers (ESRs), in order to address research with an array of methods and know-how from different fields, and more importantly to design biomimetic models for solving complex tasks;
- bringing together scientists with complementary, but distinctive interests in chemical and biological subjects. All groups gain not only from the reciprocal expertise, but also in providing a whole scenario for improving the biomimetic modeling;
- promoting contributions from different disciplines, such as analytical, radiation, mechanistic and synthetic chemistry, biochemistry, molecular biology, pharmacology, together with computational tools contributing to the understanding of free radical processes at a molecular level in order to reach an integrated vision of chemistry related to other sciences;
- addressing important societal needs related to health and technology with favorable impact, and also for clarifying the perception of chemistry's contribution to the overall progress;
- improving a common language among scientists of different background and experience, creating a common environment for sharing methodologies and solving analytical/mechanistic problems, and enhancing the importance of chemical and molecular aspects within the biological and technological scenarios.

Last but not least, the Action's participants wished to contribute actively to the scientific impact and success of the Action by an increasing number of articles on the common research. The Special Issue of Free Radical

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Research dedicated to the Action and the papers published herein demonstrate the strong intention to collaborate that animated all participants.

The relevant achievements will be described below in more detail and divided into contributions from each Working Group, thus providing an updated bibliography on the topic of Biomimetic Radical Chemistry.

### Working Group 1: radical enzymes

Radical enzymes catalyze many biological reactions, e.g. biosynthetic pathways to antibiotics and cofactors. In addition to this medical context, radical enzymes are essential for the degradation of alkanes and aromatic hydrocarbons derived not only from natural processes, but also as a result of environmental damage from oil spills and other industrially caused pollution [1]. The primary objectives of WG1 were:

- To elucidate the catalytic pathways and mechanisms of selected radical enzymes.
- To discover new radical enzymes.
- To develop model systems and biomimetic chemistry based on the pathways used by radical enzymes.

There were many issues that WG1 sought to resolve through collaborations between members of WG1 and with other Working Groups. Major advances were made in the understanding of the mechanisms of radical enzymes and were aided by computational studies. Novel potent inhibitors of selected enzymes were synthesized. The power of physical methods for determining binding interactions and elucidating reaction pathways was demonstrated.

Radical enzymes catalyze reactions in which species with an odd number of electrons, classically described as “free radicals”, participate [2]. This area has been intensively developed in recent decades and is now a major sub-discipline of enzymology.

Anaerobic degradation of alkanes is in contrast to their long-known O<sub>2</sub>-dependent oxidation by cytochrome P450 mono-oxygenase [3]. Thus, hexane is oxidized by the proteobacterium strain HxN1 in a tandem process requiring two radical enzymes: the first of which attacks a C-H bond, whilst the second effects a coenzyme B<sub>12</sub>-dependent molecular rearrangement. A putative HxN1 enzyme uses a cysteinyl radical to abstract stereospecifically the pro-S hydrogen atom from C-2 of hexane. This step may be a concerted reaction in which hydrogen atom abstraction is coupled with addition of the hex-2-yl moiety to fumarate. Computational chemistry employing density functional theory supported this

concept in an inter-Working Group collaboration that will be published in due time [L. A. Eriksson (WG2), Buckel, B. T. Golding, and D. M. Smith (Zagreb)]. Current work is focused on the analogous degradation of decane and defining the role of coenzyme B<sub>12</sub> in alkane degradation. The expertise of I. Smonou's group in stereochemically controlled enzymatic reductions of ketones [4] led, in collaboration with Golding's group, to the synthesis of (2*R*,9*S*)-decane-2,9-diol, a precursor of (2*R*,9*S*)-[2,9-<sup>2</sup>H<sub>2</sub>]decane, required for studies of anaerobic decane oxidation. In related studies, Buckel and Golding (with J. Heider, Marburg) have shown that benzylsuccinate synthase combines toluene and fumarate with inversion of configuration at the methyl group [5].

Sulfur-containing small metabolites are key players in cellular oxidative stress. F. P. Seebeck's group has studied the mechanism of enzyme-catalyzed oxidative sulfur-carbon bond formation. In the biosynthesis of aromatic thiols, e.g. thiohistidines, there is an unprecedented transfer of a sulfur atom from cysteine or  $\gamma$ -glutamylcysteine ( $\gamma$ GC) to the imidazole ring of *N*<sup>2</sup>-trimethylhistidine. The crystal structure of the iron(II)-dependent radical enzyme EgtB that is responsible for the biosynthesis of ergothioneine has been solved [6] and a catalytic mechanism proposed, in which Tyr377 acts as a general acid catalyst [7].

A. K. Croft's group initiated computations on radical SAM enzymes [8], notably the enzyme QueE, which is involved in biosynthetic pathways for antibiotics, antivirals, and anticancer agents. This work led in 2015 to the award of a Marie-Curie CASCADE fellowship and the Nottingham Research Fellowship to C. Jaeger for the project “Radical Enzymes for Biotechnology – Pioneering with Computational Chemistry”, for which Dr. Croft is host. As a result of this COST Action, inter-WG collaborations have been established with M. Davies (WG3) and H. Zipse (WG4), as well as the groups of M. Boll and M. Seemann.

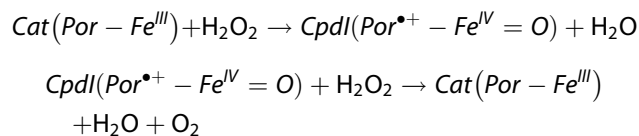
Most discussions of life's origin focus on heterolytic chemistry even though many radical processes in biology have been recognized. A computational study taking today's extant biology [ribonucleotide reductase (RNR) mechanism featuring a cysteine thiyl radical] and extrapolating back, in an attempt to identify prebiotic radical chemistry, led to the proposal that the parent thiyl radical HS<sup>•</sup> derived from H<sub>2</sub>S was the prebiotic equivalent of the cysteinyl radical of RNR [9]. Modeling of this process was initiated through collaboration between C. Ferreri (WG3) and B. T. Golding with the aid of an ESR aided by a short-term scientific mission (STSM) grant.

F. Dekker's group has developed novel small molecule inhibitors for the radical enzyme 15-LOX-1 [10],

which plays an important role in the regulation of inflammation. The aim was to inhibit proinflammatory gene transcription, with N. Rohr-Udilova (WG3) performing biological studies on the inhibitors. In addition, the inhibitors will be tested in a cellular model for hepatocellular carcinoma, as 15-LOX-1 is involved in this type of cancer. Interaction with A. Georgakilas (WG2) led to a review [11] on the role of lipoxygenases in both inflammation and cancers. Development of probes for activity-based protein profiling of the radical enzyme lysine demethylase 1 has been pursued by A. Lenoci (University of Rome “La Sapienza”), through funding of a STSM.

Mechanistic investigations of IspH/LytB, a [4Fe-4S]<sup>2+</sup> radical enzyme target for the development of new antimicrobials, have been pursued by M. Seemann’s group. In many bacteria (e.g. *Mycobacterium tuberculosis*), in plant chloroplasts and in the malaria parasite *Plasmodium falciparum*, the biosynthesis of isoprenoids proceeds via the methylerythritol phosphate (MEP) pathway, an alternative to the well-known mevalonate pathway. The MEP pathway is absent from humans and is therefore a target for the development of new selective antibacterial and antiparasitic drugs. IspH/LytB, the last enzyme of the methylerythritol phosphate pathway, converts (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) into a mixture of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In collaboration with V. Schünemann (TU Kaiserslautern), the structure of substrate-free LytB was clarified in solution [12]. Potent LytB inhibitors were designed and prepared in collaboration with D. E. Poulter (University of Utah, Salt Lake City, UT, USA), whereby the OH group of HMBPP was replaced by a thiol (TMBPP, *K*<sub>i</sub> 20 nM) or an amino group (AMBPP, *K*<sub>i</sub> 54 nM) [13].

Mechanisms of action of some catalase inhibitors have been studied by the Gebicki-Gebicka group. Catalases are ferriheme enzymes that catalyze decomposition of hydrogen peroxide to water and molecular oxygen. The catalytic cycle proceeds in two steps, with the formation of radical intermediate, compound I (*Cpd I*):



{where, *Cat* = catalase, *Por* = porphyrin}

Under some conditions, e.g. under low fluxes of H<sub>2</sub>O<sub>2</sub> and in the presence of appropriate substrate, *Cpd I* is reduced to an inactive Compound II (*Cpd II* (*Por*-Fe<sup>IV</sup>=O)). The Gebicki team has found that flavonoids, plant polyphenols, inhibit catalase *in vitro* [14,15], which

is due, at least partially, to conformational changes of the enzyme upon flavonoid binding, and the reduction of *Cpd I* to inactive *Cpd II* by flavonoids.

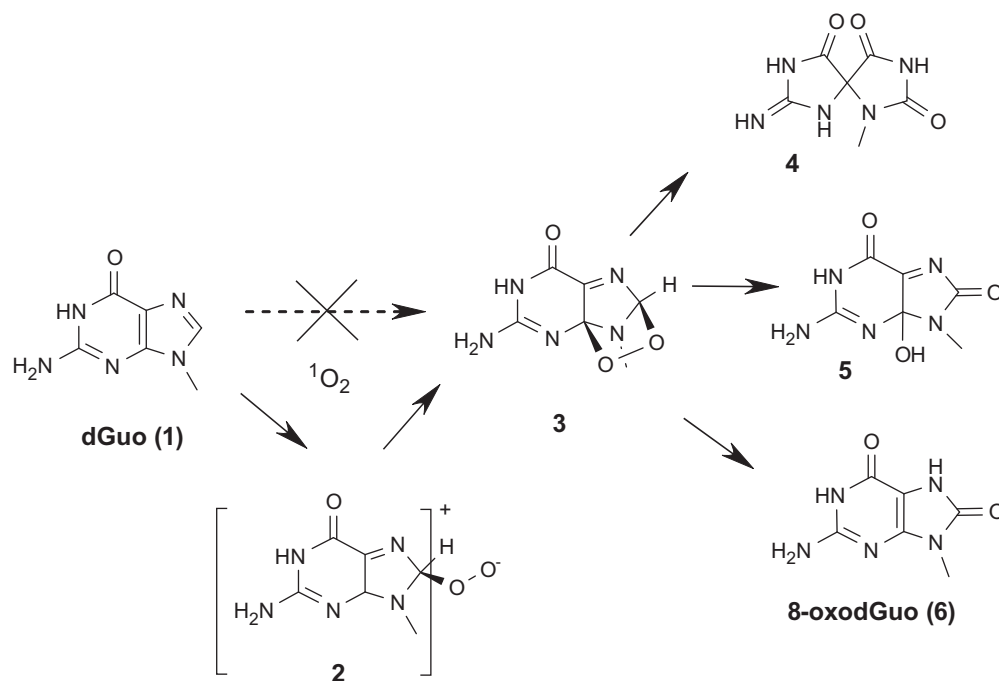
Serum albumins are a significant class of proteins in the circulatory system, which can act as carriers for a broad spectrum of compounds or assemblies such as nanoparticles. Research in G. Ionita’s group was focused on studies of protein/surfactant complexes, monitoring conformational changes induced by temperature, surfactants, and cyclodextrins, using spin labeling methods and EPR spectroscopy to investigate these systems [16]. The collective data may support potential applications in protein purification [17] or in optimizing drug formulations.

Interactions of serotonin with myeloperoxidase were investigated by the group of M. Číž. Serotonin inhibited the chemiluminescence response of neutrophils in human whole blood partially due to the direct scavenging activity of serotonin toward individual reactive oxygen species and partially due to the inhibition of myeloperoxidase activity [18]. Serotonin acts as an alternative substrate for myeloperoxidase and therefore can undergo oxidation via the peroxidase cycle of myeloperoxidase. Recent data suggested that myeloperoxidase activity differs according to the pH value.

## Working Group 2: models of DNA damage and consequences

Due to its important role in the maintenance of the genetic information, any modification of the DNA macromolecules may have harmful effects. Hence, the study of DNA damage formation, its efficacy of repair, and subsequent biological consequences are a matter of great interest. Among the genotoxic agents that could damage DNA, ionizing radiations and photosensitization reactions could induce the formation of DNA radicals that can be subsequently converted into chemical modifications of the DNA structure. Several radicals, having very short lifetimes, could be produced giving rise to a plethora of different DNA lesions. Thus, several experimental and theoretical approaches have to be used and combined to better delineate the mechanisms of formation of these DNA lesions. Moreover, to understand the effects of the modifications produced, collaboration with biologists is also essential. This was one of the main objectives of Working Group 2 and is illustrated here by the presentation of several examples of the results obtained by the different partners of this Working Group.

Reaction of singlet oxygen with DNA has been studied in detail during the last two decades [19]. Experimentally it has been shown that <sup>1</sup>O<sub>2</sub> reacts



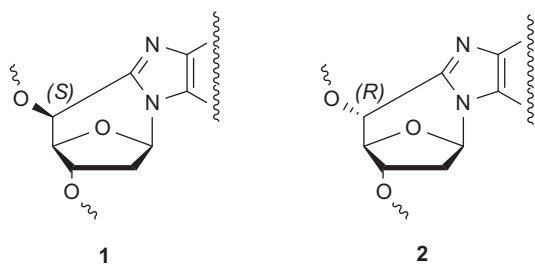
**Scheme 1.** Proposed mechanisms for the reaction of singlet molecular oxygen ( $^1\text{O}_2$ ) with the guanine moiety of DNA.

exclusively with the guanine moiety of DNA [20]. Several guanine decomposition products can be formed (Scheme 1) and interestingly the reactivity is different for the free nucleoside (2'-deoxyguanosine in aqueous solution) compared to what is observed in double-stranded DNA. The postulated mechanism for the formation of the decomposition products involves first the formation of a guanine endoperoxide (3, Scheme 1) through a [4 + 2] Diels-Alder cycloaddition of  $^1\text{O}_2$  onto guanine (1) [20]. Such an intermediate has been observed experimentally at low temperature [21]. Its decomposition generates spironucleosides (4), 4-hydroxy-8-oxo-2'-deoxyguanosine (5) and 8-oxodGuo (6) as final stable products (Scheme 1). At the nucleoside level, spironucleosides are the major products, whereas in double-stranded (ds)DNA, 8-oxodGuo is predominantly generated.

A couple of experimental results were difficult to explain. First, if  $^1\text{O}_2$  reacts with dGuo through a Diels-Alder reaction with the imidazole moiety of that purine base, why does such a reaction not take place with adenine, the other purine base having a similar imidazole ring? Second, why is the reactivity of  $^1\text{O}_2$  with guanine different in DNA compared to what is observed for the free nucleoside in aqueous solution? Theoretical approaches have been used to decipher the reaction mechanisms of singlet oxygen with DNA and to compare its reactivity with guanine and adenine [22,23]. The results show that if indeed the endoperoxide is generated by reaction of singlet oxygen with guanine; its

formation does not proceed through a Diels-Alder reaction, since  $^1\text{O}_2$  first adds to the C8 position of guanine to produce a zwitterionic intermediate (2). A higher energy barrier has been calculated for reaction with adenine, giving for the first time an explanation of the lack of reactivity of  $^1\text{O}_2$  toward adenine. Moreover, the attack of singlet oxygen onto 8-oxodGuo has been found to have only a very low barrier, rationalizing the experimentally observed higher reactivity than guanine itself [25]. The results obtained highlight the importance of theoretical studies [24,25] to delineate the mechanisms of formation of DNA lesions that have been identified experimentally.

Theoretical approaches have been also used to model the interaction between DNA and external molecules potentially acting as photosensitizers [26–38]. This has allowed the recognition of different competitive modes, by means of classical all-atom molecular dynamics, and the subsequent elucidation of the different photochemical pathways [26,27]. Modeling of electronic circular dichroism also allows for a straightforward relation with experience, also in the case of noncanonical DNA structures such as G-quadruplexes [28,32]. In particular, we have established the presence of a double-inserted interaction mode for benzophenone, and relying on QM/MM methodologies, we have also evidenced the mechanisms of triplet transfer toward DNA thymine [35]. Finally the use of nonadiabatic dynamics has allowed unravelling of the time scale and the electronic mechanisms leading to the triplet population in



**Scheme 2.** Structure of the two diastereomeric forms of 5'-8-cyclopurine nucleosides.

benzophenone despite the relative weak spin-orbit coupling, i.e. the first step of photosensitization [30]. We have also analyzed the possibility of other photochemical reactions such as hydrogen abstraction [31], evidencing strong environmental effects and a global self-protection by DNA. In the same spirit, we have analyzed electron transfer, in the case of Nile red and Nile blue sensitizers, showing once again a marked effect of the DNA environment [33]. Also the photophysical mechanisms leading to the environmental controlled production of singlet oxygen by the alkaloid palmatine only upon DNA interaction have been rationalized as mainly due to the differential stabilization of charge-transfer excited states [26]. Finally sensitization by artificial nucleobases mimicking DNA lesions has also been assessed.

The research of WG2 has also been focussed on complex DNA lesions that may represent a challenge for the DNA repair machinery. Among them, 5',8-cyclo-2-deoxyadenosine (cdA) and 5',8-cyclo-2-deoxyguanosine (cdG) are tandem lesions produced by hydroxyl radicals ( $\text{HO}^\bullet$ ) of endogenous or exogenous origin that insult DNA by abstracting a hydrogen from the C5 of a purine nucleoside moiety [39]. Both cdA and cdG lesions having a C5'-C8 bond exist in two diastereomeric forms, 5'S and 5'R (Scheme 2).

There were many issues related to cdA and cdG lesions that WG2 addressed through collaborations between its members and associated scientists from USA. Major advances were made in the synthesis of two diastereomers of cdG and their incorporation into oligonucleotides [40]. Lipophilic derivatives of cdG have been used to study the association constant with cytidine [41] and formation of G-quadruplex in chloroform [42]. Spectroscopic data indicated that the Watson-Crick complex of cytidine with cdG is weaker than with guanosine, the difference being one order of magnitude between the two association constants [41]. A "fully-anti" lipophilic G-quadruplex, a dodecameric complex composed of three stacked G-quartets of 5'S-cdG in the presence of strontium picrate, was observed for the first time [42].

A cost-effective and efficient protocol for the quantification of the four purine 5',8-cyclo-2'-deoxynucleosides together with the two 8-oxo derivatives (8-oxo-dA and 8-oxo-dG) in oxidized DNA was obtained [43] and applied to radiation-induced formation of purine lesions [44-46]. In single- and double-stranded DNA, the overall lesion formation follows the order: 8-oxo-dG  $\gg$  8-oxo-dA  $>$  5'R-cdG  $>$  5'R-cdA  $>$  5'S-cdA  $>$  5'S-cdG [44]. The rate constants for a unimolecular pathway were estimated to be ca.  $5 \times 10^4 \text{ s}^{-1}$  for both 2'-deoxyadenosin-5'-yl and 2'-deoxyguanosin-5'-yl radicals in ss-DNA, and about halved ( $2-3 \times 10^4 \text{ s}^{-1}$ ) in ds-DNA [44,45]. The same purine lesions were also quantified in order to individuate the influence given by different DNA helical topology and folding on the purine exposure to the damage [46]. This was investigated in tertiary dsDNA helical forms of supercoiled (SC), open circular (OC), and linear (L) conformations, along with single-stranded folded and non-folded sequences of guanine-rich DNA in selected G-quadruplex structures. Purine oxidation was indeed different following the order: L  $>$  OC  $\gg$  SC, indicating greater damage toward the extended B-DNA topology. In G-quadruplex sequences, the unfolded states were more resistant compared to G-tetrad folded topologies.

Major advances were also made in understanding the biology of these lesions by incorporating them in DNA sequences. Both cdG and cdA lesions, in their 5'S and 5'R diastereomeric forms, are repaired at low efficiency by the human nucleotide excision repair (NER) pathway, there being ca. 2 times greater efficacy for 5'R over 5'S diastereomer repair, owing to greater DNA backbone distortions encountered in 5'R diastereomeric lesion-containing sequences [47,48]. Given the known correlation among oxidative DNA damage and its repair in trinucleotide repeats (TNR), it was investigated whether TNR instability can be induced by the cdA lesions during replication and repeat [49]. Evidence that a cdA can effect TNR deletion by inducing the formation of various sizes of loop structures with the lesion in the template strand of a TNR repeat tract were obtained. DNA pol  $\beta$  efficiently bypasses 5'R-cdA, but inefficiently bypasses 5'S-cdA during DNA replication and base excision repair (BER) [50]. This result highlights that the diastereomeric nature of purine cyclonucleoside lesions can play a crucial role in DNA repair mechanisms.

Even though much work has been done during the last three decades to determine the nature of the modifications produced in DNA exposed to ionizing radiations, we could not totally exclude that still unknown lesions are produced in cells. In particular, much attention has been focused on complex DNA lesions

produced by a single radical [51], as described above for cyclonucleosides. These lesions could be produced by reaction of the initially generated radicals with surrounding molecules and in particular with other DNA bases producing intra-strand crosslinks and also tandem lesions (for a review see [52]). In cellular DNA, the situation is even more complicated since DNA is covered by several proteins, in particular histones, and also other small molecules such as polyamines that play an important role in the regulation of several biological functions. Previous works have shown that the guanine radical cation ( $G^{\bullet+}$ ), that is the main one-electron oxidation product of DNA due to an efficient charge transfer reaction in dsDNA, reacts with water to produce 8-oxodGuo. Moreover, addition of other nucleophilic molecules [53], including for example lysine to produce guanine-lysine crosslinks [54], has also been reported. In addition, it has been also shown that the presence of tyrosine could chemically repair  $G^{\bullet+}$  to restore guanine [55,56], by an electron transfer reaction from tyrosine to  $G^{\bullet+}$ . This suggests that in cells, the fate of the initially produced guanine radical cation will depend on its environment. Since polyamines, including spermine, spermidine, and putrescine are found at very high concentration in the nucleus of eukaryotic cells (mM range) the possibility that these nucleophilic molecules add to  $G^{\bullet+}$  has been studied. It has been shown *in vitro* that such addition is very efficient [57], and that the presence of these polyamines (at concentrations below 1 mM) completely inhibits the formation of 8-oxodGuo that is compensated by the formation of polyamine-dGuo adducts. The reactivity of the guanine radical cation has been used to determine that UV-induced formation of oxidative DNA lesions involves a one-electron oxidation reaction and not singlet oxygen as initially reported [58]. Additional work has now to be done to determine the importance of these lesions at the cellular level. This requires the development of a highly sensitive and specific method that could quantify levels of modifications lower than one lesion per million nucleosides, using a few microgram of DNA. Nowadays, HPLC coupled through electrospray ionization to tandem mass spectrometry represents the most powerful method that has already been applied to monitor several lesions in cellular DNA exposed to oxidative stress [59].

However such methods do not allow localization of the lesion within the DNA sequence, and following one-electron oxidation reactions, the DNA sequences play a predominant role. Indeed, the redox properties of DNA are of outstanding importance for understanding the site distribution of DNA oxidative damage and for clarifying mechanistic aspects of long-range hole transfer.

In that context, the possible establishment of charge delocalized domains is of particular significance, in as much as delocalized domains are expected to exhibit lower oxidation potentials, changing hole site energies and speeding up hole migration along the strand. In a collaborative research differential pulse voltammetry (DPV) has been carried out on several guanine-rich oligonucleotides, containing up to six consecutive guanines. The progressive lowering of the first oxidation potential as the number of adjacent guanines increases unambiguous points toward the establishment of delocalized hole domains [60]. Simulations of hole transfer dynamics in double-strand G(T)nG oligonucleotides, performed by using a set of parameters inferred from electrochemical measurements, have clearly shown the effects of delocalized domains on hole transfer rates: for small  $n$ , hole transfer rates exponentially depend on the donor-acceptor distance, whereas for  $n > 3$ , the rates become almost independent of distance. That behavior has been assigned to the formation of hole delocalized domains in the complementary strand, which by increasing  $n$  become nearly degenerate with the donor state [61].

DPV measurements have also been used to investigate the redox properties of 5',8-cyclo-2'-deoxyguanosine (cdG), the DNA tandem lesion referred to above. Electrochemical measurements have shown that the interaction energies of the damaged nucleoside with cytosine differ from that of undamaged base. Indeed, the equilibrium association constant, determined by NMR titrations, is found to be ca.  $10^3 \text{ M}^{-1}$ , about one order of magnitude less than that for guanosine [41].

In addition to complex lesions produced by a single radical, ionizing radiations produce lesions in cluster, also called multiple damage sites (MDS), constituted of several modifications (including oxidized bases, strand breaks, abasic sites, etc...) within one or two helix turns. When LET (Linear Energy Transfer) increases the complexity (number of individual lesions in one multiple damage site) increases, since more ionization events take place in a defined volume. In a close collaboration with Iliakis' (Germany), Ravanat's (France), and Martin's (Australia) groups, the DNA Damage Laboratory of A. Georgakilas (NTUA, Greece) has proposed an improvement of methodologies used for the detection of oxidative clustered DNA lesions *in vitro* and *in vivo* with special emphasis on the *in situ* detection including bystander effects [62–65]. In addition, bioinformatics has been used to better understand the DNA damage response mechanisms relating to ionizing radiation-induced DNA damage and their association with inflammatory and immune response pathways [66–71]. Collaboration between the groups of Monari and



Dumont (France), and the Georgakilas group, through the application of Molecular Dynamics (MD) simulations, gave mechanistic insights on the processing of clustered DNA lesions especially for cases where it is very difficult to acquire analytical experimental data [72,73]. By using classical molecular dynamics, we have analyzed the position and sequence-dependent structural modification induced on B-DNA by the presence of a bistranded clustered abasic lesions. In particular, we have seen that the deformation may lead to the loss of the abasic lesions signature, hence preventing recognition by a repair enzyme such as bacterial Nfo. On the contrary, human repair enzymes such as APE1 exert much stronger mechanical constraints and are characterized by higher repair rates. There are a few exceptions that have been examined in these papers. In addition, through the use of Monte Carlo (MD) simulations, a special biophysical model was developed for the simulation of radiation-induced systemic effects and the interpretation of various nontargeted effects [67]. Last but not least, in a series of publications, Georgakilas' group within its participation in the network of the Halifax Project contributed toward the designing of a broad-spectrum integrative approach for cancer prevention and treatment as reviewed in [73,74]. Overall, such a work within this COST Action has been the development of a solid mechanistic basis for the induction and propagation of oxidative clustered DNA damage in tissues and organs as the result of ionizing radiation exposure or tumor development, where oxidative stress is involved. Special emphasis was given in the delineation of cellular and organism mechanisms implicated in the confrontation of complex DNA damage and the result of deficient repair [75].

Since DNA is a target of several medical approaches aiming at killing undesired cells, for example for cancer therapy (using chemo- or radio-therapeutic approaches, as well as photodynamic therapy), increasing our basic knowledge in this field would certainly allow to improve the efficacy of such treatments.

One of approaches that help to overcome the hypoxia-related radioresistance of tumor cells is to substitute their DNA with sensitizing nucleosides. This is why the reactivity of halogenated 2'-deoxynucleosides (brominated (BrdX) or iodinated (IdX)) toward organic radicals and solvated electrons (both types of species are produced during the radiolysis of cellular environment) was investigated in the current project.

Using HPLC and LC-MS techniques, it was demonstrated that beside the substitution of bromine atom by 2-hydroxypropyl radical (OHisop<sup>•</sup>), electron transfer from OHisop<sup>•</sup> to BrdX takes place only for 5-bromo-2'-deoxyuridine (BrdU) and 5-bromo-2'-deoxycytidine

(BrdC) due to larger electron affinity (EA) of pyrimidines than purines [76]. The larger EA of brominated pyrimidines also accounts for more efficient damage induced by hydrated electrons in the trimeric oligonucleotides, TBrdXT, labeled with BrdU or BrdC [77]. Moreover, the extent of damage, especially the formation of cytotoxic single-strand breaks induced by hydrated electrons is doubled in the trimeric oligonucleotides labeled with IdU or IdC compared to their counterparts labeled with BrdU or BrdC [78]. Our studies showed unequivocally that hydrated electrons are a negligible factor in damage to native DNA even for a ionizing radiation dose exceeded many times the doses used in radiotherapy [77]. Therefore, some kind of radiosensitization seems to be an indispensable component of any efficient radiotherapy.

In WG2, the Kellet laboratory focused on the development of nucleic acid-interacting inorganic materials for biochemical and therapeutic application. Since the discovery of the first chemical nuclease, [Cu(phen)<sub>2</sub>]<sup>+</sup> (where phen = 1,10-phenanthroline), this agent has served as a template for the rational design of new DNA oxidants and has been widely applied as a footprinting reagent. Recently, we reported [Cu(phen)<sub>2</sub>]<sup>+</sup> as a novel agent for protein engineering when applied under Fenton-type conditions against a recombinant antibody fragment specific for prostate-specific antigen (PSA) for the generation of recombinant mutagenesis libraries. This study was highlighted as a front cover article in Chemical Communications and may now open up new applications for inorganic DNA oxidants [79]. By structurally modifying this chemotype, bis-chelate Cu(II) phenanthroline-phenazine complexes [Cu(DPQ)(phen)]<sup>2+</sup>, [Cu(DPPZ)(phen)]<sup>2+</sup> and [Cu(DPPN)(phen)]<sup>2+</sup> were recently reported to improve DNA intercalation and sequence selectivity [80]. This complex series displayed high-affinity DNA binding at both the major and minor grooves, where the most active derivatives (DPQ and DPPZ) demonstrated the highest known binding constants for Cu(II) complexes at the time of publication. DNA oxidation was further quantified through an "on-chip" microfluidic protocol [80]. Further mechanistic investigation of this class, reported in collaboration with the Chatgililoglu group [81], revealed the DNA cleavage mechanism was linked to metal-oxo or free hydroxyl (<sup>•</sup>OH) radical production that produced the oxidative lesion 8-oxo-dG. Metal-catalyzed oxidative DNA damage of oligonucleotide templates were further found to inhibit the polymerase chain reaction (PCR); this reaction was particularly impeded within A·T transcripts [81]. Since copper(II) phenanthroline complexes also possess therapeutic potential, the anticancer activity of a square planar

dicarboxylate complex [Cu(o-phthalate)(phen)] was recently reported [82]. Submitted to the National Cancer Institute (NCI) 60 cancer cell line screen, the complex exhibited broad-spectrum anticancer activity by exerting mitochondrial and nuclear DNA damage via superoxide-mediated ROS production [82]. The Kellett group has also pursued platinum(II) drug research in collaboration with the group of N. P. Farrell: fully trans-symmetric tri-platinum(II) (Triplatin) complexes with varying aliphatic diam(m)ine linkers were reported [83]. The architecture of this chemotype facilitates a noncovalent surface interaction called the “phosphate clamp” that results in two binding motifs: backbone tracking and groove spanning. This work revealed the two limiting modes of phosphate clamping are sequence selective and could be distinguished in solution through conformational changes. Triplatin-DNA binding also prevented endonuclease activity by blocking the action of site-selective type II restriction enzymes [83].

The above reported results obtained by participants of the WG2 highlight the importance of networks bringing together scientists having different and complementary expertise and working in a similar field of interest, such as radiation-induced damage to DNA. Exchanges of ideas or experimental/theoretical competences during presentations and discussions through frequent meetings organized by such a network promote collaborations between physicists, chemists, and biologists who usually do not have the opportunity to discuss together. Multidisciplinary approaches are required to better understand the effects of radiation on DNA, keeping also in mind that cells are constituted not only of nucleic acids but also of lipids and proteins. In addition, such a network facilitates the involvement of young scientists who represent the future of the European research community.

### **Working Group 3: membrane stress, signaling, and defenses**

Membrane organization as a phospholipid double layer and its chemical and biochemical reactivity attracted more research interest starting from the 1990s, as shown by a Web of Science search [84]. However, the importance of cell membrane formation given by spontaneous phospholipid assembly and the connection between phospholipid structures and functions were already well understood in the 1970s, as explained by the fluid mosaic model of Singer and Nicolson [85]. Only many years later a larger view of the phospholipid molecules, reactivity, and consequences started to be addressed by appropriate research, in particular evidencing free radical reactivity with lipids as a crucial

process to occur under stress conditions, accompanying other biochemical and chemical stress processes regarding all cell components. The activity of Working Group 3 was directed toward an interdisciplinary approach to address the research described below.

### ***Lipid peroxidation, membrane signaling, and antioxidant mechanism***

Lipid peroxidation mainly involves polyunsaturated fatty acids (PUFA) and leads to their transformation and degradation [86]. One of the interesting products is the family of  $\alpha,\beta$ -unsaturated aldehydes, the 4-hydroxyalkenals [e.g. 4-hydroxynonenal (4-HNE)], which derive from the omega-6 reactivity, as well as two other members of this family, 4-hydroxy-2E-hexenal (4-HHE) and 4-hydroxy-2E,6Z-dodecadienal (4-HDDE) [87,88]. They are chemically reactive, forming covalent adducts with amino or thiol moieties in proteins, phospholipids, and nucleic acids, and the toxicity of the adducts can determine different pathological and cytotoxic processes. However, there is also an important signaling role for these aldehydes that was discovered studying beta pancreatic cells after glucose stimulation. In fact, 4-HNE signaling stimulates the cell response for insulin production, after liberation of the precursor omega-6 fatty acid from cell membranes and transformation by peroxidation [89–91]. Another interesting molecular mechanism was discussed in the Working Group, determining that by the interaction of these aldehydes with amino-containing membrane phospholipids (phosphatidyl ethanolamine, PE) lipid adducts are formed, with consequences for the transmembrane translocation process of proton and potassium ions [92].

The research of Working Group 3 has clearly evidenced membrane participation in stress processes, which include signaling and other biological consequences. Indeed, a large number of signaling cascades start from the detachment of fatty acids from membrane phospholipids and this phenomenon causes a profound membrane fatty acid remodeling for the phospholipid replacement, known as Land’s cycle [93]. This natural remodeling cycle accompanying each cascade is an opportunity for membrane shaping and controlled phospholipid replacement following the availability of fatty acids in the organism. The WG3 expertise of several groups (Rome Catholic University, Hebrew University of Jerusalem, Consiglio Nazionale delle Ricerche, University of Patras) provided a multidisciplinary environment that led to an understanding of remodeling (evaluated by fatty acid-based membrane lipidomics and analysis of fatty acid metabolites such as 4-HNE) and its consequences on membrane physical

parameters (evaluated with biophysical methods) in two case studies: pancreatic beta-cells exposed to different glucose and palmitic acid concentrations, and red blood cells of genetic mouse models defective in lipoproteins [94]. The importance of the membrane molecular variations associated with signaling cascades will be surely deepened in further research focused on inflammatory and metabolic processes. This result will trigger also new perspectives in the fields of nutritional and physiochemical conditions to control cell membrane compositions, which can contribute to the control of degenerative processes that accompany stress and related responses.

The role of lipid peroxidation as a free radical-mediated mechanism has been better and better understood, nowadays seen not only as damage caused to cells and their membranes, precisely by the reaction of unsaturated fatty acid moieties, but also as a trigger of cellular stress response. This implies also the activation of enzymatic defenses and the role of antioxidants, in a complex balance involved in pathological conditions such as inflammation, as well as in the progression of the aging. The Working Group 3 of the Action was very active on this aspect. In particular, the antioxidant mechanism was thoroughly studied by Valgimigli's group as related to anthocyanins [95] and to the redox balance connected with longevity [96]. In the field of antioxidants, some studies addressed analytical detection of oxidative stress in humans. New methods have been reported such as a noninvasive EPR methodology [97], as well as antioxidant evaluation for *in vitro* activity [98].

The evaluation of the protection from oxidative stress given by a number of vitamins and cofactors in animal models and humans was also addressed in the WG3 activities. A collaborative research between Italy, Greece, and France was related to a mitochondrial-targeted antioxidant used in rats fed an obesogenic diet [99], establishing positive protective effects on the polyunsaturated moieties of cardiolipin, the typical lipid of mitochondria. The model of diabetic rats was used to investigate the effect of SOD-mimics and L-arginine on skin injuries, based on the correction of the ratio between superoxide radical anion and nitric oxide, proving that restitution of redox balance can be an approach for the resolution of such dermatological problems [100].

Children affected by autism spectrum disorders (ASD) were considered for their levels of thiamine and related phosphorylated metabolites in plasma and urine in a collaborative study between Warwick and Bologna Universities. A significant decrease (24%) of thiamine pyrophosphate in the patients was evidenced compared to controls [101].

Determination of antioxidant status in diseases was addressed in a collaborative paper between Akdeniz University of Antalya, Turkey and University of Modena and Reggio Emilia, Italy. They studied the dynamic thiol/disulfide homeostasis in prostate cancer patients. Indeed, patients showed less thiol and total antioxidant status (TAS) than controls and there was a negative correlation between the prostate specific antigen (PSA) and thiol levels [102].

### **Lipid isomerization**

The cis-trans isomerization process discovered in 1999 occurs by free radicals adding reversibly to lipid double bonds, thus transforming the naturally occurring cis geometry of unsaturated lipids into the thermodynamically more stable trans configuration, without shifting the double-bond position [103]. In this process, the bent molecular shape of the cis isomer is converted into the almost straight structure of the trans isomer. This molecular conversion has been an important focus of Working Group 3 and review articles summarized the state-of-art in the field [104]. Collaborative research in the COST Action was directed toward the details of isomerization processes in different biomimetic models, such as liposomes and micelles.

Micelles formed by linoleic acid were studied for their reactivity under irradiation, in aerobic and anaerobic conditions, in the presence of aromatic amines and thiols [105]. Depending from the reaction conditions, isomerization and peroxidation processes could be examined and compared, confirming that both processes contribute to the fatty acid changes under free radical conditions, as previously detected only with thiol [106]. In the presence of amines, the cooperative production of thiyl radicals was evidenced, that contributes to increase the cis-trans double-bond conversion. The isomerization vs. peroxidation processes were also evaluated in this system in the presence of antioxidants such as resveratrol, ascorbic acid, and alpha-tocopherol, finding that trapping of the initiating radicals, generated by ionizing radiation in the water phase, and co-localization of the antioxidant at the hydrophobic core of the micelle are necessary for an effective inhibition of lipid degradation [107]. In liposomes made of phospholipids with unsaturated fatty acid residues, the isomerization process was also evaluated in detail, comparing also different system of liposome formation (injection vs. extrusion methodologies), as well as the inhibition effect of the most common antioxidants [108].

### **Fatty acid-based membrane lipidomics applied to erythrocyte membrane mapping**

In the activities of the COST Action CM1201, the training of early stage researchers (ESR) was an important objective achieved by learning protocols to examine the membrane effects at the level of fatty acid residues in phospholipids. This included a thorough training in protocols for the isolation and analytical determination of fatty acids.

The WG3 activities, involving the work of 3 ESRs, led to the discovery of a new fatty acid biosynthetic transformation that occurs in human metabolism. Fatty acid biosynthesis is known to start from palmitic acid (hexadecanoic acid, C16:0), obtained by *de novo* synthesis, that is subsequently converted to palmitoleic acid (9-*cis*-hexadecenoic acid, C16:1) by the intervention of stearoyl CoA-desaturase enzyme. An increase of this metabolism has been correlated to diseases such as obesity and cancer. In the research developed in this COST Action, a new conversion of palmitic acid was discovered that is carried out by the delta-6 desaturase, normally operating with PUFA. In the case that delta-6 desaturase works on palmitic acid, it leads to the formation of a monounsaturated fatty acid, that is positional isomer of palmitoleic acid, namely sapienic acid (6-*cis*-hexadecenoic acid, C16:1) [109]. The presence of such isomer was not known previously in human blood, and this was a discovery carried out in the frame of the COST Action. After setting-up a protocol for the effective separation and recognition of fatty acids in plasma, red blood cell and lipoprotein lipids, the presence of positional and geometrical (*cis* and *trans* isomers) isomers of C16:1 was achieved applying specific conditions for the full resolution by gas chromatography of the hexadecenoic fatty acid family. This research arose an important awareness for lipidomic studies concerning the identification of positional and geometrical isomers possessing the same molecular weight, that are nowadays proposed as biomarkers in several diseases. Subtle differences of mass fragmentation percentages might be difficult to evidence or interpret, especially in biological samples where lipids are mixed and in low quantities. Therefore, a combined approach was studied to corroborate mass spectrometry analysis with other structure determination methods to arrive to an unambiguous determination. The research in this COST Action research achieved a high resolution protocol for positional and geometrical isomers. An important application was the analysis of fatty acid components of red blood cell membrane phospholipids and plasma cholesteryl esters in healthy human subjects and morbidly obese patients, revealing that

sapienic acid is an interesting biomarker of metabolism diversion in obese subjects versus controls. These findings are important for deepening biological studies of lipid enzymes, such as delta-9 and delta-6 desaturases as well as LCAT (lecithin cholesterol acyl transferase), that are certainly key steps of metabolism differentiation in health and disease [110].

In a collaborative research between Italy and Argentina, fostered by the COST Action environment, the erythrocyte membrane lipidome in infertile men was considered in a representative group of 11 subjects. Significant differences with healthy population were evidenced, especially highlighting an unbalanced membrane status, with loss of important components such as omega-3 fatty acids. After a supplementation of these fatty acids effected for 3 months, a second analysis on plasma and sperm membrane lipids was carried out thus evidencing that changes of membrane lipidome occurred mostly in the erythrocytes, with increased levels of the supplemented fatty acids [111]. This study is also important for nutraceutical applications, suggesting to approach by membrane lipidomics the bioavailability of supplemented or dietary fatty acids for sperm cells.

Another issue addressed by the WG3 research in lipidomics was the importance of building up integrated panels with other molecular methodologies, such as for example, the measurement of DNA damage, foreseeing the use for diagnostics taking into account the full monitoring of physio-pathological status of cells related to diseases. Using auto-modified poly(ADP-ribose)polymerase (PAR-PARP), a well-known early DNA damage sensor, and erythrocyte membrane fatty acid composition of 95 subjects with different health conditions, the statistical correlation between disease presence and alteration of these molecular indicators was evaluated. Statistical significant correlations were found in some conditions between these two indicators, generally in agreement with the knowledge that PARP activation is connected with upregulation of proinflammatory gene expression for which PUFA are the signaling molecules. In particular, positive correlation was found between PAR-PARP levels and DHA (omega-3 fatty acid) in thyroid diseases; in Hashimoto's disease, increased EPA (omega-3 fatty acid) correlated positively with PAR-PARP levels; in patients affected by allergy, PAR-PARP corresponded to an increase of DGLA (omega-6 fatty acid) and DHA levels; in patients affected by HCV (Hepatitis C virus), PAR-PARP levels were inversely correlated to EPA and DHA values [112].

### **Free radical biomimetic and biological models**

In the frame of the interdisciplinarity of free radical research in biosciences, that is one of the main objectives of the COST Action CM1201, a combination between biomimetic and biological models was successfully obtained by a collaborative work on the mechanism of the bleomycin-induced phospholipid transformation [113,114]. Bleomycin is an antitumoral drug, with a well-known mechanism of intercalation in the DNA sequence and generation of hydroxyl radicals that cause DNA strand break. In a collaboration in the WG3 between the Ferreri (Italy) and Ozben (Turkey) groups, it was found for the first time that bleomycin is able to damage membrane lipids, both via inducing peroxidation and, in the presence of thiols, isomerization processes. The mechanism and influencing factors were tested in biomimetic models of liposomes, containing mono- and poly-unsaturated fatty acids. In parallel, testicular cancer germ cells were cultured with bleomycin, showing that lipid peroxidation and isomerization do occur as seen in the models. Also the signaling related to such transformation has been examined in some detail.

Another biomimetic model that has been studied in the collaborative environment of WG3 was related to the generation of radicals on peptide sequences, in particular to understand the pathways of transformation involving sulfur-containing moieties. The role of methionine (Met) as amino acid and in peptides was thoroughly studied by the group of Chatgililoglu, and in this COST action the Gly-Met-Gly sequence and its N-acetyl derivative, which simulate the “internal” disposition of Met, were used for an interdisciplinary approach, including kinetic, mechanistic, computational, and product studies [115]. Significant differences of the radical generation and fate were detected compared to previous studies carried out with free Met. Mechanistic pathways and a full scenario of the reactivity were obtained by pulse radiolysis detection of transient species, radiolytical, and electrochemical transformations of the tripeptide and a detailed product analysis.

The 4 years of collaborative research of Working Group 3 evidenced the crucial role of membrane lipids and membrane processes in the free radical transformations of biological importance, and this increased knowledge will certainly be relevant for a large scientific audience. These achievements acquired an important applicative meaning, due to the participation of companies in this Working Group, which were also as co-author of some papers [90,110,112]. The presence of companies certainly contributed to the discussion of results in terms of scientific innovations that have to be

brought to the market for amelioration of the products and improving benefits to consumers.

The final goal of Working Group 3 dedicated to the membrane field was to create a bridge between chemical models and biological systems for the study of the influence of membrane organization and structures. This included the effects of chemical and biochemical transformations, taking into account that the main signaling activities depart from membranes with cascades involved in metabolic processes such as stress, inflammation, and aging, as well as the protection given by specific compounds with antioxidant/anti-isomerizing properties. The two main lipid transformations, peroxidation and isomerization, and the lipid remodeling were focused upon and the Working Group discovered several new aspects of the fatty acid influence on the favorable recovery of the whole membrane functioning and homeostatic balance. Actually bringing the aspect of membrane lipidomics into molecular sciences was thoroughly discussed among the Action participants, aiming at having a complete scenario of the cellular activities, taking account of the undoubted role of cell membranes for replication and life.

Discussion of the above reported results with the other Working Groups brought about very productive interactions and new collaborations, thus creating innovative research networks that can apply to the Horizon2020 calls.

### **Working Group 4: bio-inspired synthetic strategies**

Working Group 4 was concerned with the development of bioinspired synthetic organic strategies having free radicals as central intermediates. Nature uses free radicals to accomplish notoriously difficult biosynthetic transformations to produce the molecules it needs for signaling, metabolism, and host defense. To accomplish that, Nature applies, on one hand, enzymatic catalysis to bring about these transformations very specifically. On the other hand, spontaneous free radical-based events, such as autoxidative or single-electron transfer processes occur frequently. Both of these free radical-based pathways have implications for the design of synthetic strategies. In contrast to Nature, which applies free radicals in a very specific environment, organic chemistry is ideally suited to probe the generality of free radical chemistry. This means that a much larger chemical space can be covered by synthetic endeavors. Small-molecule catalysts can be discovered and varied in wide margins, may they be organic or metal-based. Light can be used to trigger radical generation, biomolecules may be selectively modified by free radical C-H

functionalization, and radical reactivity can be modulated by the electronic and steric properties of substituents. This leads to new insights into the physical parameters of free radical reactions. Here, collaboration with computational chemists is very important for providing rationales for the observed reactivity patterns. This reasoning formed the basis for the collaboration among the Working Groups in the COST action and has been amply demonstrated by the results obtained by the members of the Working Group as illustrated below.

The synergistic combination of expertise was accomplished in an inter-working group mode, as documented by the collaborative work of the Ferreri and Lykakis groups, who studied the biomimetic thiyl radical chemistry [105,106] and the protection of phospholipids from thiyl radicals by antioxidants [107,108], as explained previously in the Working Group 3 activities. Another important collaboration concerned the investigation of iron-based 1-aminocyclopropane-1-carboxylic acid oxidase mimics, whose kinetic and mechanistic features were experimentally and computationally determined [116].

A major effort in WG4 was directed toward the discovery of catalytic radical reactions. The Ollivier, Goddard, and Fensterbank group reported photoredox-catalytic Barton-McCombie reactions as an alternative to the traditional method mediated by toxic tributyltin hydride [117]. Inspiring dual photoredox- and nickel-catalyzed cross-coupling reactions of benign alkylsilicates provided interesting products [118]. The Lykakis group investigated heterogeneous catalysts and especially the photocatalytic activity of mesoporous ternary polyoxometalate-Ag<sub>2</sub>S-CdS semiconductors [119]. The Jahn group provided the first examples of oxidative electron transfer-catalyzed polar-radical crossover reactions, in which the necessary stoichiometric sacrificial oxidant is at the same time an oxygenation reagent and allows more atom-economic reactions [120]. The Speier/Kaizer group reported bioinspired organocatalytic oxidations of thiols [121] as well as 1,3,2-oxazaphosphole catalysts as flavin mimics [122].

The power of radical chemistry to contribute to the development of green and sustainable chemistry was demonstrated. The Speier/Kaizer group reported the use of greenhouse gas CO<sub>2</sub> as an oxidant and a C1 building block in organic chemistry [123]. A green method for the oxidation of alcohols using heterogeneous photocatalysis was developed by the Lykakis group [124].

The potential of changing the properties of biomolecules by radical chemistry is another focus in the Working Group as exemplified by an approach to

selective oxidative intramolecular modifications of cyclodextrins [125].

Last, but not least, organic synthesis with the help of free radical was significantly developed in the action. Such approaches open up new avenues for medicinal chemistry to increase the potential of three-dimensional biologically active entities. The Renaud group described a radical cascade reaction for the preparation of pyrrolidine derivatives, using thiyl radical addition to a terminal alkyne function as the first step, which was followed by a 1,5-hydrogen transfer, translocating the radical center for the subsequent cyclization to afford the heterocyclic ring [126]. The Herrera group demonstrated that normally hard to functionalize methyl groups can be easily activated for cyclizations to pyrrolidines by the iodine/iodobenzene diacetate reagent system [127]. Antiviral cyclopentane derivatives became easily accessible by new complex tandem reactions consisting of polar organometallic additions coupled with radical cyclizations and oxygenations [128,129] and a new approach toward the total synthesis of bridged diketopiperazine alkaloids using the persistent radical effect emerged [130]. Alternatively, the central diazabicyclo[2.2.2]octane core of several alkaloids can be approached by reaction sequences proceeding through single electron transfer (SET) oxidation steps, thus switching among multiple intermediate types of different oxidation state in one pot [131].

The importance of free radical processes in stereoselective synthesis for the transformation of enantiomerically pure substrates without racemization was also addressed in the WG4 research, as beautifully reviewed by Renaud's group [132].

These examples document the central role of organic synthesis based on free radicals to develop more efficient processes, to enable new and more benign approaches to organic compounds, new methodology for medicinal and material chemistry, as well as to provide new tools and standards for biology.

## Conclusions

The summary of the activities carried out by the COST Action Working Groups demonstrates the successful implementation of the proposal to bring free radical research at the cross-road of life and material sciences, and the capability of scientists to interact efficiently and productively for the ultimate benefit of the proposed research. Many of the results obtained have been inspired and influenced by the discussions at meetings and subsequent collaborations, partly through STSM grants. The interdisciplinary environment created by the

COST Action has already given results in bilateral or multilateral proposals presented to funding agencies.


The success of the COST Action initiative was due also to the extremely efficient and wise management provided by the Chairman Chrysostomos Chatgililoglu, the Vice Chairman Philippe Renaud, as well as by the Grant Holder and the COST Office staff.

### Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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