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Chemical Mutagenesis

J.M.Leitão*

BioFIG, FCT, Universidade do Algarve, 8005-139 Campus de Gambelas, Faro, Portugal
* Corresponding author, E-MAIL: jleitao@ualg.pt, jleitao2@gmail.com

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1. Introduction

The first attempts to induce mutations in biological systems using chemical compounds go back to the beginning of the past century. However, it was during World War II that the two most relevant names in chemical mutagenesis, Charlotte Auerbach and Iosif A. Rapoport, established the mutagenic properties of several chemical compounds (**Box 12.1**). A detailed review of these and other major moments in the history of plant chemical mutagenesis is given by van Harten (1998, **see Chapters 1 and 2**).

There is currently an enormous number of known chemical compounds able to induce mutations in prokaryotic and/or eukaryotic cells and this continues to increase. The continuous search and the synthesis of new mutagenic compounds is driven, not by the needs of experimental mutagenesis, but by the paradoxal fact that several mutagenic compounds, although carcinogenic, possess simultaneously anti-neoplastic properties and find application in anti-tumour therapy.

Despite the large number of mutagenic compounds, only a small number has been tested in plants. Among them, only a very restricted group of alkylating agents has found large application in plant experimental mutagenesis and plant mutation breeding. Over 80% of the registered new mutant plant varieties reported in the IAEA database (http://mvgs.iaea.org/Search.aspx) obtained *via* chemical mutagenesis were induced by alkylating agents. Of these, three compounds are significant: ethyl methanesulphonate (EMS), 1-methyl-1-nitrosourea (MNU) and 1-ethyl-1-nitrosourea (ENU), which account for 64% of these varieties (**Figure 12.1**).

2. Alkylating Agents

2.1. Sources and Types of Alkylating Agents

Alkylating agents are strong mutagenic, carcinogenic and cytotoxic compounds (**Figure 12.2**). Paradoxically, the cytotoxic properties of some of the compounds are largely exploited in cancer therapy. Alkylating agents can be found among a large panoply of classes of compounds, including sulphur mustards, nitrogen mustards, epoxides, ethyleneimines and ethyleneimides, alkyl methanesulphonates, alkylnitrosoureas, alkylnitrosoamines, alkylnitrosoamides, alkyl halides, alkyl sulphates, alkyl phosphates, chloroethyl sulphides, chloroethylamines, diazoalkanes, etc.

Although most are synthetically produced, a few alkylating agents are of biological origin, e.g. the strong mutagenic glucosamine-nitrosourea (Streptozotocin) is produced by *Streptomyces achromogenes.*

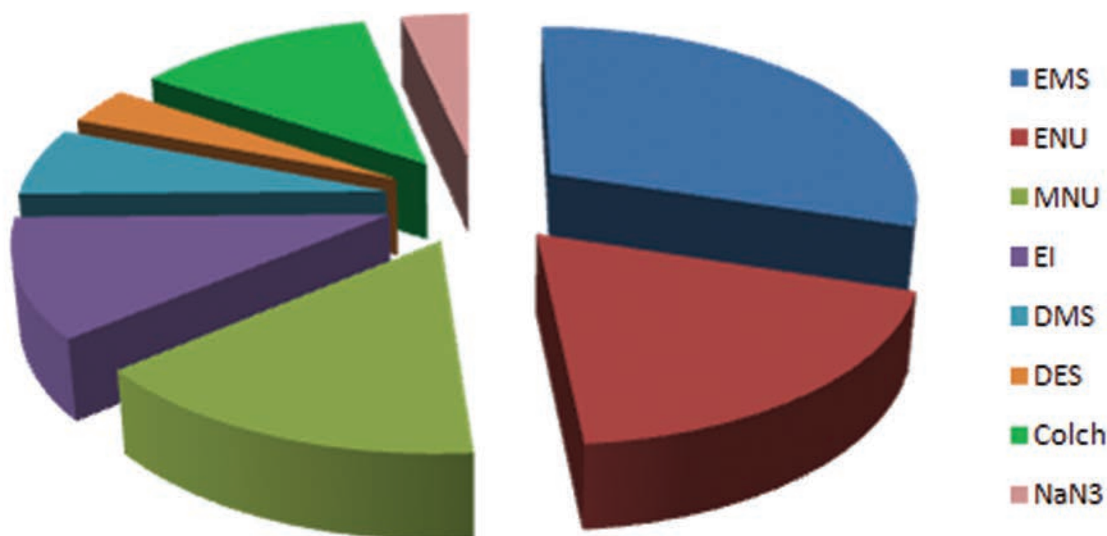

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Figure 12.1 Relative number of released mutant varieties (direct and indirect) induced using the agents indicated. EMS – ethyl methanesulphonate; ENU – 1-ethyl-1-nitrosourea; MNU – 1-methyl-1-nitrosourea; EI – ethyleneimine; DMS – dimethyl sulphate; DES – diethyl sulphate; Colch – colchicine; NaN_3 – sodium azide.

Box 12.1: C. Auerbach and I. Rapport and their work on chemical mutagenesis

Charlotte Auerbach (1899–1994) was born to a Jewish family in Krefeld, Germany. She attended classes at the Universities of Berlin, Würzburg and Freiburg. After her "State examination" in 1924 and a short period of research in developmental biology she spent some years teaching at various schools in Berlin. After Hitler became the German Chancellor and new laws prohibited Jews from teaching in state schools she moved to the UK. There, through Professors H. Freundlich and G. Barger, she was introduced to F.A.E. Crew, head of the Institute of Animal Genetics in Edinburgh, where she started to work on Drosophila and was awarded a Ph.D. in 1935. In 1939 she acquired British citizenship, thus avoiding incarceration in internment camps during the second World War.

In 1938 Crew introduced Auerbach to H.J. Müller who stimulated her to test a number of chemical agents known to be carcinogenic for induction of mutations in Drosophila. After obtaining negative results with 1:2:5:6-dibenzanthracene, 9:10-dimethyl-1:2 dibenzanthracene and methyl-colanthrene, in 1940 Auerbach began to work in collaboration with J.M. Robson on the mutagenic effects of mustard gas.

Robson had at that time already observed the antimitotic effect of this compound in the vaginal ephithelium of rats. During their collaboration most of the experimental treatments were carried out by Robson and his collaborators, while Auerbach performed the genetic analysis of treated Drosophila using the ClB method introduced by Müller, with whom she discussed experimental results.

In 1942, Auerbach and Robson sent several reports to the Ministry of Supply of the British Government where they described the induction of sex-linked lethal, chromosomes inversions and translocations by mustard gas (encrypted as substance "H"). They also reported mustard gas to act directly on the chromosomes while describing the induced visible mutations and discussing the similarities and dissimilarities of the effects of this chemical mutagen and those of X-rays.

In a letter to *Nature* in 1944, Auerbach and Robson mentioned that they had tested a number of chemical substances for their ability to produce mutations and that some of them were very effective, producing mutation rates of the same order as those with X-rays. However, it was not until 1946 in a new letter to *Nature* that the name of the mutagenic substance, dichloro-diethyl-sulphide or mustard gas, was disclosed. According to Auerbach the idea of testing mustard gas was suggested by the pharmacologist A.J. Clark who saw similarities between the long-lasting effects of this compound and that of X-rays, and hypothesized mustard gas to have effects on the genetic material.

Auerbach kept working in the field of mutagenesis, even long after she retired, publishing several articles and some major books, some of them translated into multiple languages. She was honoured with many prizes and distinctions including the Darwin Medal by the Royal Society in 1977.

Iosif Abramovich Rapoport (1912–1990) was born in Chernigov, Ukraine. In 1930 he started his studies at the Faculty of Biology at the Leningrad State University and in 1938 he was awarded a Ph.D. in biological sciences by the Institute of Genetics of the Academy of Sciences. A Doctor of Sciences degree was awarded in 1943 after he defended his thesis and while attending a rapid course for commanders at the Frunze Military Academy in Moscow.

Twice injured during the war (he lost an eye) Rapoport was awarded many major military medals of the Soviet Union and the USA Legion of Merit. After the war he resumed his research at the Institute of Cytology, Histology and Embryology in Moscow, working on chemical mutagenesis in Drosophila.

Although the first mutants induced by chemical mutagenesis were probably identified by V.V. Sacharov in 1932, the early work of Rapoport led to the identification of a number of mutagenic agents: formaldehyde, urotropine, acrolein, ethylene oxide, ethyleneimine, diethylsulphate, diazomethane and N-nitrosomethylurethane. As with Auerbach, Rapoport used Müller's ClB method to assess mutagenic effects in Drosophila. The first article on these discoveries: "Carbonyl compounds and chemical mechanisms of mutations" was published in 1946 and several other papers were published until 1948.

As an opponent to Lysenkoism and refusing to recognize his "error" Rapoport was excluded from the Communist Party and from 1949 to 1957 he worked as a paleontologist and stratigrapher. In 1957 (Stalin died in 1953) Rapoport joined the Institute of Chemical Physics of the Academy of Sciences in Moscow where he resumed his work on chemical mutagenesis.

Rapoport established the mutagenic properties of 55 chemical compounds including the nitrososureas, which he called "super mutagens" and still are largely used in plant chemical mutagenesis today. A centre for introduction of chemical mutagenesis in the biosynthetic (biotech) industry and agriculture was created in 1965. Headed up by Rapoport this centre had a tremendous impact in the utilization of the chemical mutagenesis in the Soviet Union, eastern European countries and other countries. Numerous mutant varieties were officially released in these countries and registered in the IAEA database; 383 chemically induced mutant varieties of major crop species were released in the former USSR alone by 1991.

Later in his life Rapoport was awarded the most prestigious social and academic distinctions of the USSR.

References:

Beale, G. (1993) The discovery of mustard gas mutagenesis by Auerbach and Robson in 1941. *Genetics.* 134: 393–399.

Some alkylating agents such as the methyl-donor S-adenosylmethionine (SAM), which in spite of being involved in about 40 metabolic reactions in mammalians is a weak methylating agent able to form adducts to DNA, are formed endogenously as natural products of organisms. Other alkylating agents such as chloromethane (CH_3Cl), formerly thought to be uniquely of ocean origin but today assumed to have a dominant terrestrial origin as result of the reaction of plant pectin with chloride, are naturally formed in the environment. A significant source of environmental exposure of humans to alkylating compounds, particularly to the carcinogenic 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and N'-nitrosonornicotine (NNN), is tobacco smoke.

Alkylating agents are electrophilic compounds with affinity for nucleophilic centres in organic macromolecules to which they bind covalently. In DNA these compounds form covalently linked alkyl adducts to the bases and to the phosphodiesters.

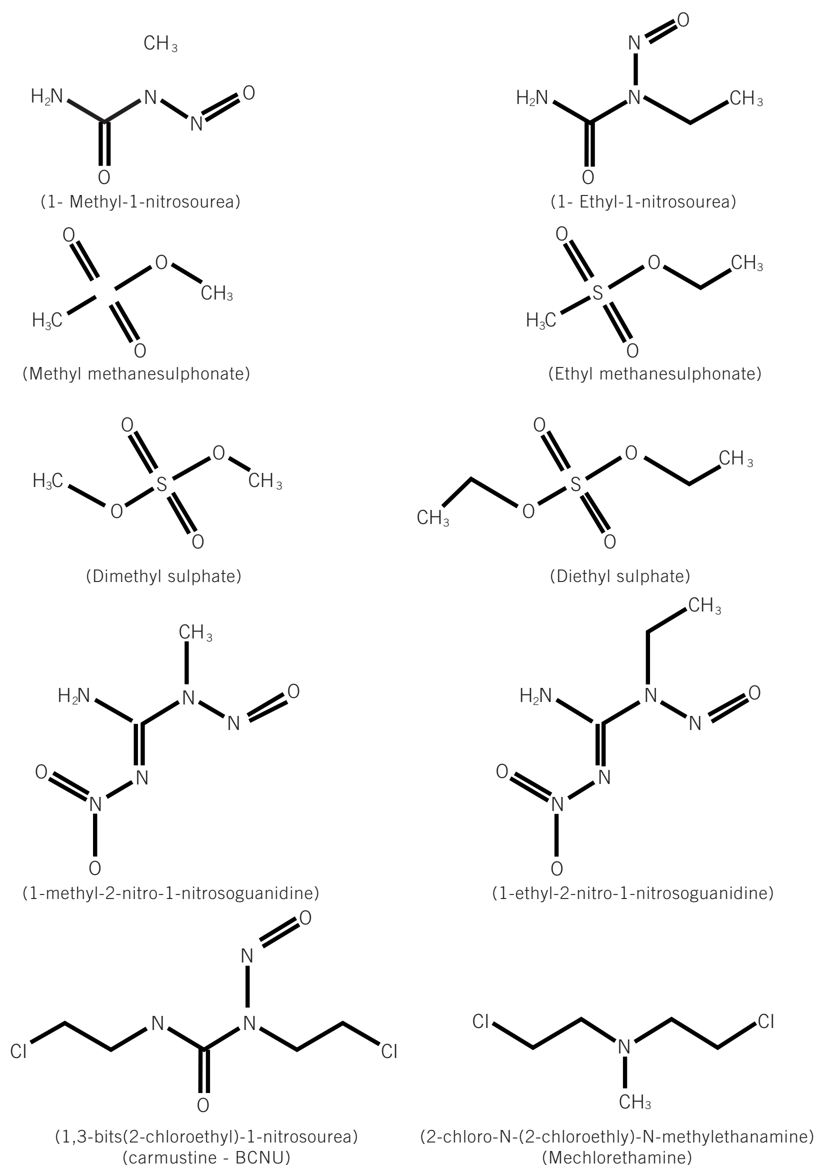

Figure 12.2 Molecular structure of commonly used alkylating agents in plant mutagenesis, and carmustine and mechlorethamine, two bi-functional alkylating agents used in anti-neoplastic clinical practice.

Despite some controversy, alkylating agents are usually classified as S_N1-type or S_N2-type according to their kinetic properties. Compounds for which the rate determining (slow) step in the alkylation reaction is a first-order kinetics formation of reactive intermediates independent of the substrate (in our case–DNA) are designated S_N1-type alkylating agents. Those compounds, where the rate-determining step is a second-order nucleophilic substitution reaction involving both the compound and the substrate (DNA) are designated S_N2-type alkylating agents (**Figure 12.3**).

The very reactive electrophilic species produced by the S_N1-type alkylating agents are generated independently of the substrate and are relatively unselective towards the nucleophile they alkylate. Consequently, these compounds alkylate nitrogen, oxygen and phosphate group sites in DNA. The S_N2 compounds, where the substrate (DNA) participates directly in the generation of the reactive species, react primarily with the most nucleophilic sites on DNA, i.e. the nitrogens N7 and N3 of guanine and N3 of adenine. (**Figure 12.4, Table 12.1**)

Although most of the commonly used alkylating agents in plant experimental mutagenesis produce similar spectra of alkylation lesions in DNA (**Figure 12.4**), differences in alkylation mechanisms (S_N1 vs S_N2) give rise to differences in the proportions of lesions produced.

Alkylation products	MNU	ENU	MMS
O^2-alkylcytosine	< 1	< 1	< 1
N^3-alkylcytosine	< 1	< 1	< 1
N^3-alkylguanine	< 1	< 1	< 1
O^6-alkylguanine	6	~ 7	0.2
N^7-alkylguanine	65	~ 13	83
O^2-alkylthymine	< 1	~ 5	< 0.1
N^3-alkylthymine	< 1	~ 1	< 1
O^4-alkylthymine	< 1	~ 5	< 0.1
N^1-alkyladenine	2	~ 1	1
N^3-alkyladenine	7	~ 6	11
N^7-alkyladenine	2	~ 1	2
Phosphotriesters	20	~ 65	1

Table 12.1: Alkylation products of major representative S_N1 (MNU, ENU) and S_N2 (MMS) compounds [a]

[a] Data kindly provided by Prof. A. E. Pegg, Dep. of Cellular and Molecular Physiology, Milton s. Hershey Medical center, Pennsylvania State University College of Medicine, Hershey Pennsylvania 17033, USA

For example, 1-methyl-1-nitrosourea (MNU) and 1-ethyl-1-nitrosourea (ENU) react *via* an S_N1 mechanism and efficiently alkylate both nitrogens and oxygens in DNA. Methyl methanesulphonate (MMS), which reacts *via* an S_N2 mechanism, predominantly alkylates the nitrogens at the DNA bases and produces little alkylation of the oxygens in DNA bases and in the sugar–phosphate backbone (**Figure 12.4; Table 12.1**).

Alkylating agents are commonly divided into two classes: mono-functional and bi-functional. The alkylating agents usually used in plant experimental mutagenesis and mutation breeding are mono-functional. Bi-functional alkylating agents (**Figure 12.2**) such as the chloroethylating agents (e.g. 1,3-bis(2-chloroethyl)-1-nitrosourea–BCNU), the nitrogen mustards or mitomycin C, are characterized by their ability to induce DNA strand cross-links and are utilized as anti-neoplastic compounds. This last class of alkylating agents has not been utilized in plant mutation breeding experiments.

2.2. Properties of the Alkylating Agents

The physical and chemical properties of the alkylating agents and of innumerous other chemicals, as well as

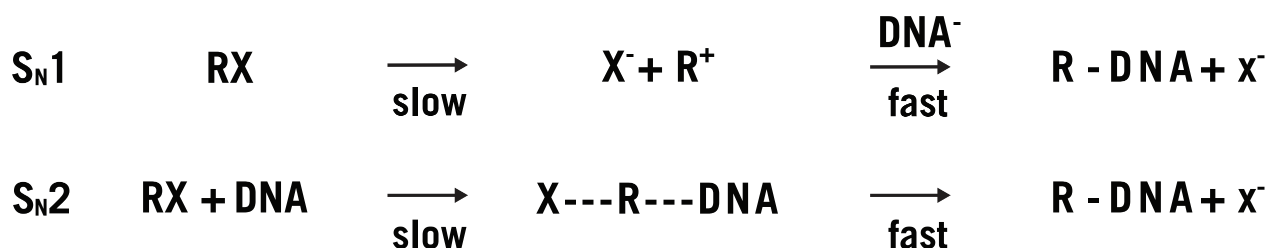

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Figure 12.3 The S_N1 and S_N2 mechanisms of alkylation.

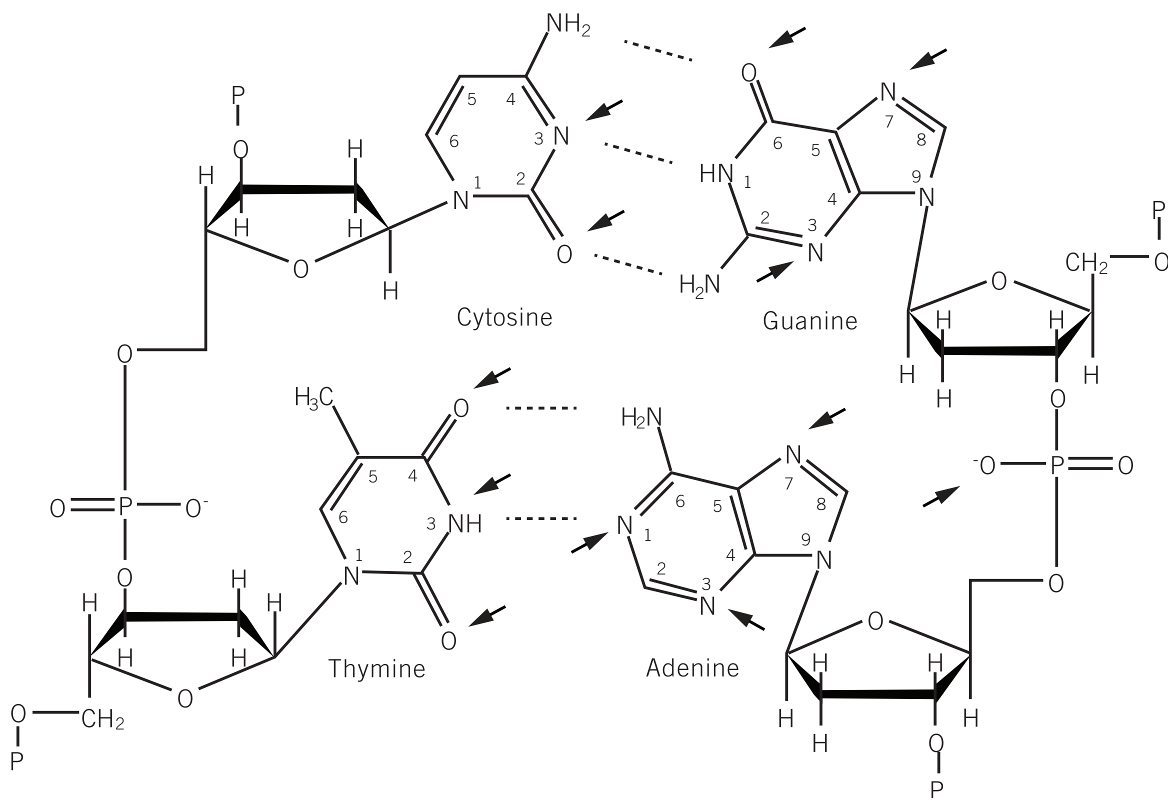

Figure 12.4 Most frequently alkylated sites in DNA.

information regarding their biological activity, chemical structure, and other properties, can be retrieved from the Pubchem database (http://pubchem.ncbi.nlm.nih.gov). The Substance Identity and the Compound Identity codes for some alkylating agents are given in **Table 12.2**. Both codes can be used interchangeably after accessing the database provided that the respective links "Substance" or "Compound" are previously selected.

Many other databases are available, inter-linked and inter-active. For example, the Toxicology Data Network (http://toxnet. nlm.nih.gov) links over a dozen different databases (including Pubchem). Broad and regularly updated and peer-reviewed information regarding multiple aspects of the compounds, from physical and chemical properties to biological activity and risk, handling, clean-up, disposal, etc., can be found in these databases. **Box 12.2** shows a partial example of the information that can be retrieved from Toxnet – in this case showing information regarding the inactivation and disposal of solid waste and mutagenic solutions after 1-methyl-1-nitrosourea treatments. Some general rules for inactivation of alkylating compounds are provided simultaneously.

2.3. Major Alkylation Adducts and their Repair

Eleven sites in the four bases and the phosphodiester groups constitute the 12 most common targets for the alkylating agents in DNA (**Figure 12.4**). Nevertheless, additional minor sites can be identified, such as the exocyclic 2-amino group of guanine in which alkylation by the bi-functional agent mitomycin C was recently determined.

N^7alkylguanine – the most nucleophylic site in DNA, the N^7 position of the guanine is the primary alkylated site in DNA. Although this represents the bulk of the DNA alkylation by most alkylating compounds, this altered base is, apparently, non-mutagenic.

O^6alkylguanine – a major characteristic product of the S_N1-type alkylating agents *O^6*-alkylguanine is strongly mutagenic since it mispairs with thymine and gives rise to G:C – A:T transitions. The repair of this lesion is particularly crucial in humans, given that besides the mutagenic implications there is a very well established strong correlation between the formation of this adduct and carcinogenesis. This DNA lesion is repaired in many prokaryotes and eukaryotes through a direct

Table 12.2: Commonly used alkylating agents in plant mutagenesis and mutation breeding

Compound IUPAC name	Acronym(s)	Substance[a] identity (SID)	Compound[b] identity (CID)
1-methyl-1-nitrosourea	NMU (MNU)	24897498	12699
1-ethyl-1-nitrosourea	NEU (ENU)	24897681	12967
methyl methanesulphonate	MMS	49815676	4156
ethyl methanesulphonate	EMS	24896575	6113
dimethyl sulphate	DMS	24893559	6497
diethyl sulphate	DES	24859256	6163
1-methyl-2-nitro-1-nitrosoguanidine	MNNG	49855726	9562060
1-ethyl-2-nitro-1-nitrosoguanidine	ENNG	77654587	5359974
N,N-dimethylnitrous amide	NDMA	24897656	6124
N,N-diethylnitrous amide	NDEA	49855498	5921

[a, b] Use the SID or the CID code to find the mutagen in the Pubchem database, respectively under Substance or Compound, to retrieve information regarding chemical structure, properties, safety, and other information about the compounds.

reversal and suicidal mechanism by O^6alkylguanine-DNA-alkyltransferases, which transfer the alkyl group from DNA to a cystein of the active site, which results in the inactivation of the repair protein.

The recent sequencing of the *Arabidopsis* genome revealed the absence of O^6alkylguanine alkyltransferase gene homologues in this plant species. The absence of similar gene homologues in other plants may explain the negative experimental results obtained regarding this repair activity in some plants (and in *Chlamydomonas*). Additional and alternative mechanisms for the repair of this adduct have been recently identified (**Figure 12.5**). One of the mechanisms implicates the product of alkyl-transferase like (ATL) genes in the repair of this lesion *via* nucleotide excision repair (NER).

O^4alkylthymine – usually a minor lesion, even for SN1 compounds, this altered base can mispair with guanine and lead to A:T–G:C transitions. Directly repaired by *Ogt* and *Ada* (two different O^6-alkylguanine-DNA-alkyltansferases) gene products in bacteria this lesion is very inefficiently repaired by the mammalian O^6-alkylguanine-DNA-alkyltansferase protein (known as MGMT or AGT). It should be noted that 1-ethyl-1-nitrosourea tends to induce more A:T–G:C transitions than the related compound 1-methyl-1-nitrosourea (**Table 12.3**), which is in accordance to the higher alkylation rate of the O^4 position of the thymine by the ethylating (vs. methylating) compounds (**Table 12.1**).

N^3alkyladenine – although induced by all alkylating agents this altered base is a major product of the S_N2-

Table 12.3: Mutation spectrum of the alkylating agents at the molecular level

Compound	G:C – A:T (%)	A:T – G:C (%)	Transvertions (%)	Other
MNU	~100	-	-	-
ENU	72	21	6	-
MMS	20	14	66	-
EMS	93	1	2	4
NDMA	90	< 5	-	-
NDEA	60	21	9	10
MNNG	98	< 2	-	-
ENNG	95	5	-	-
DMS	74	3	20	3

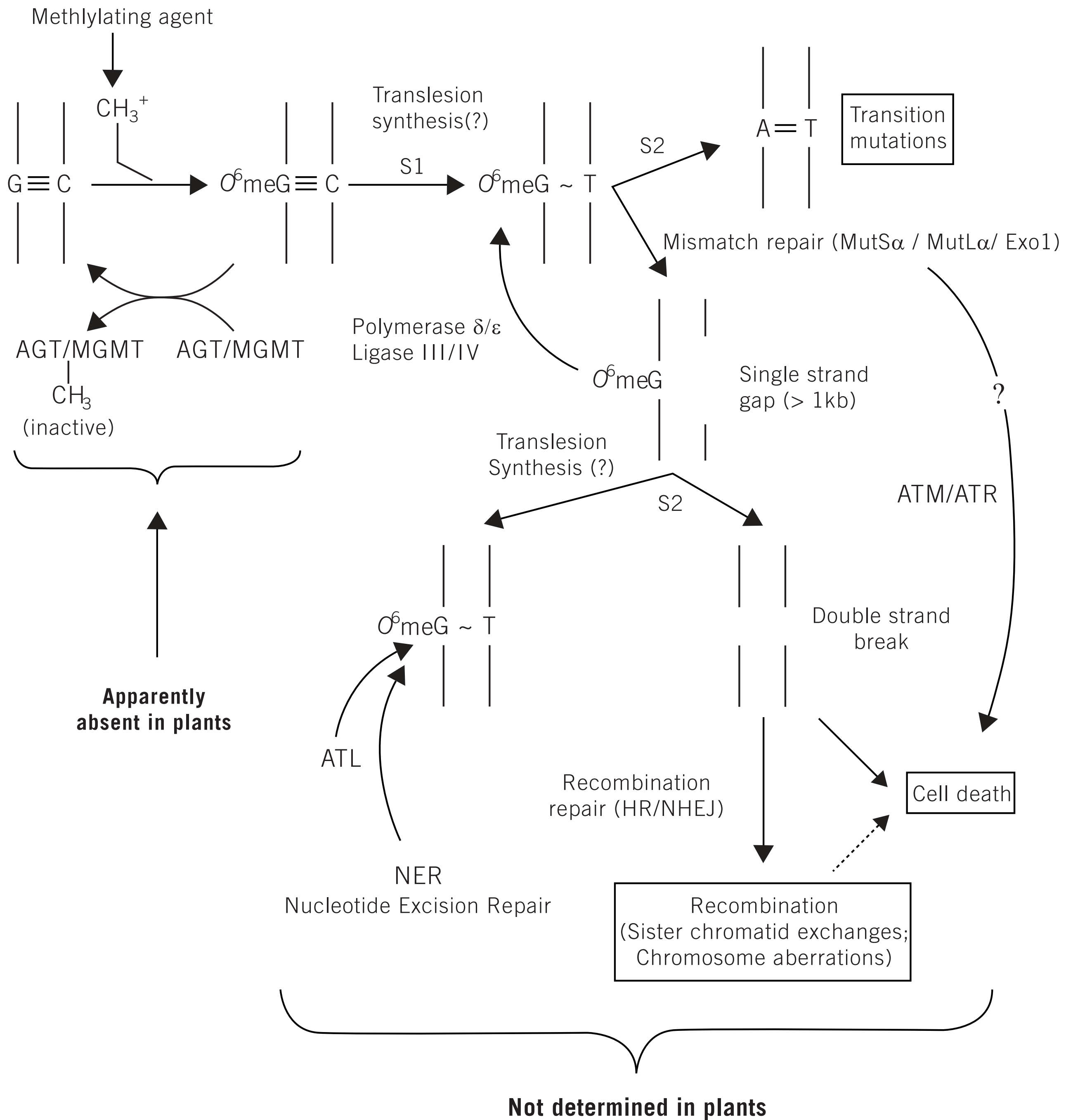

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Figure 12.5 During DNA replication, thymine can be incorporated opposite O^6-alkylG if this lesion is not repaired by the direct reversal mechanism *via* MGMT/AGT proteins. The O^6-methylG:T mispair can be recognized by the mismatch repair (MMR) machinery, but the excised thymine is replaced again by thymine resulting in a "futile" repair cycle which can lead, on a further round of DNA replication, to the induction of DNA DSBs. DSBs can be repaired by the HR or non-homologous end joining (NHEJ) pathways, but can also result in clastogeny, SCE formation and/or apoptosis. Nucleotide excision repair (NER), a possible mechanism for repair of larger adducts, can, in some organisms, be recruited by alkyltransferase like (ATL) proteins to repair O^6-methylG, possibly even before S1. S1 and S2: first and second rounds of DNA replication after alkylation. Adapted from: Margison *et al.* (2002) Mutagenesis. 17: 483–487.

type compounds. This lesion and that of N^3alkylguanine is repaired by the base excision repair (BER) mechanism, which initiates the removal of the altered base by a glycosylase activity that generates an abasic site in the double helix. Methyl methanesulphonate (MMS), an inducer of these two lesions, has been used for the study of this DNA repair mechanism in the various biological systems.

N^1alkyladenine and N^3alkylcytosine – these two minor cytotoxic lesions are also preferentially induced by S_N2 agents, which could explain the increased frequency of transversion mutations after MMS treatments. These two adducts were recently shown to be repaired by a direct reversal mechanism involving oxidative DNA demethylases (*Alk*-B in *E. coli*). In contrast to the *O^6*alkylguanine-DNA-alkyltransferase, homologues to the *Alk-B* gene have been already identified in *Arabidopsis thaliana*.

Alkylphosphotriesters – the alkylation of phosphodiesters in DNA results in alkylphosphotriesters, which in *E. coli* are repaired by the Ada-protein while in mammals they are assumed to be very poorly or even not repaired by the alkyltransferase protein (MGMT/AGT). It is noteworthy that for the S_N1 compounds MNU and ENU the alkylphosphotriesters account for approximately 20% and 70% of all alkylation adducts, respectively, and that there is an almost negligible amount of this lesion induced by the S_N2 compound MMS (**Table 12.1**).

The relative frequency of induction of a specific pre-mutagenic lesion in DNA depends mostly on the properties of the mutagen, e.g. S_N1 vs. S_N2 compounds or methylating vs. ethylating agents. The ultimate mutagenic effect of a chemical agent, however, depends on the lesions initially induced in the DNA, the lesions that remain unrepaired and the mutagenic effect of the repair mechanisms themselves.

When not repaired, the pre-mutagenic adducts tend to give rise to fixed mutations. The alkylated *O^6*guanine tends to produce G:C to A:T transitions. The alkylated N^3adenine gives rise to A:T to T:A transversions, while the non-repaired alkylated N^3cytosine can result in C:G to T:A transitions and C:G to A:T and C:G to G:C transversions.

The elucidation of the fate of the alkylation products in plants constitutes one of the main challenges for plant experimental mutagenesis. The clarification of these processes will allow a better understanding of the mutational processes that determine success in plant mutation breeding.

3. Mutagenesis of Alkylating Agents in Plants

3.1. Mutagenicity

3.1.1. DNA Breakage and Clastogenicity

Despite the relative absence of experimental data on the fate of the formed DNA adducts, the mutagenicity and the clastogenicity of alkylating agents in plants has been documented for more than 40 years. The study of the clastogenic effects of alkylating agents and other chemical compounds in plants had, and still has, two main goals: 1) the assessment of several aspects of plant cell biology related to the plant response to chemical compounds, and 2) the study of the biological activity of chemical compounds using plants as biological assay in order to draw conclusions concerning the risk they represent to the environment and human health (e.g. risk of carcinogenicity). The sensitivity and reliability of the plant bioassays have been recognized by various prestigious international organizations such as the World Health Organization, which recommend their use for mutation screening and for detection of genotoxins in the environment.

Among the multitude of plant species used for such purposes, *Crepis capillaris, Lycopersicon esculentum, Pisum sativum* and *Zea mays* were the subjects of comprehensive and informative revisions (e.g. Grant and Owens, 2001). From these and other sources it is clear that all major alkylating agents (EMS, ENU, MNU, DES, etc.) show a positive clastogenic effect in all tested plant species. Effects can be seen at the cytological level and recorded as bridges and fragments in anaphase and telophase cells, as translocations, deletions, insertions, inversions, ring chromosomes, etc. in metaphase C cells, and as micro-nuclei in interphase and mitotic cells.

Regarding the clastogeny induced by alkylating agents in plants, it was found that prior treatment of root meristems with low doses of these compounds (or with environmental stress factors as heat-shock or heavy metal salts) induced a protective (reducing) effect against subsequent treatments with higher doses of alkylating agents. By analogy with the bacterial "adaptive response" to alkylating agents, this phenomenon was named "clastogenic adaptation".

The molecular basis of "clastogenic adaptation" in plants was studied in correlation with the formation

and removal of O^6alkylguanine (Baranczewski *et al.,* 1997). Nevertheless, the apparent absence of the repair protein O^6alkylguanine-DNA-alkyltransferases in plants requires all former experimental data on "clastogenic adaptation" to be re-thought in the light of this new information. New explanations, including the role of other O^6alkylguanine repair mechanisms, are required.

DNA breakage is assumed to be the phenomenon that underlies the formation of chromosome aberrations and chromosome rearrangements. During the last two decades the assessment of genotoxic and clastogenic effects of chemical compounds in plants has been complemented with the direct assessment of DNA breakage, primarily using the single cell gel electrophoresis (SCGE) technique also known as the "Comet assay" (**see Chapter 11**).

While simultaneously evaluating DNA damage and somatic mutations in leaves of *Nicotiana tabacum*, Gichner *et al.* (1999) ranked the mutagenic potency of the four main alkylating agents as: MNU > ENU ≈ MMS > EMS. With respect to DNA damaging activity the rank order was slightly different: MNU > MMS > ENU > EMS. The reliability of the Comet assay in assessing DNA damage in different plant species is well established and a clear correlation between the extent of DNA damage and the concentration of the mutagen used has been observed in a wide range of species, including sugar beet, alfalfa, tobacco, lentil, maize, potato, durum wheat and bread wheat (Gichner *et al.,* 2003). A correlation between "clastogenic adaptation" expressed as a reduction of chromatid type aberrations, micro-nuclei and aneuploid cells, and the "clastogenic adaptation" expressed as a reduction of damaged DNA assessed by the "comet assay", was observed after the treatment with non-toxic doses of cadmium chloride prior to the challenge treatment with a high dose (5 mM, 2 h) of MNU.

3.1.2. Nature of Induced Mutations

Chemical mutagens in the early studies were termed "radiomimetic" due to the similarity of their effects compared with the radiation effects on biological systems. To the best of our knowledge, an extensive comparative analysis of the total spectra of mutations induced by radiation vs. chemical agents has never been carried out in plants. Nevertheless, in spite of differences in genome organization or cell cycle phase-dependent differences in chromatin packing that could determine differences in the accessibility of chemical compounds to DNA, the ultimate DNA lesions induced by chemical mutagens: transitions, transversions, deletions, insertions, inversions, DNA single- and double-strand breaks and DNA recombination are similar, though in different proportion, to those induced by radiation. The spectrum of mutations induced by chemical and physical mutagens, in particular the so called "visible", "macro" or phenotypic mutations (chlorophyll, morphological, physiological) is expected to be similar.

Some mutations such as those conferring increased (plastome encoded) herbicide or antibiotic resistance seem to be more frequent in chemical (alkylating compounds) mutagenesis. However, the identification of these mutations may be simply the result of the applied selection procedures.

A major difference between chemical and physical mutagenesis is the possibility of achieving higher mutations rates with minimal effects on survival and fertility of M_1 plants by chemical mutagens, a situation that does not seem possible with acute treatments of physical mutagens.

3.2. Plant Materials and Methods of Treatment

3.2.1. Types of Plant Material

Chemical mutagenesis can be performed with all types of plant materials, from whole plants (usually seedlings) to *in vitro* cultured cells. Nevertheless, the most commonly used plant material is seed. Multiple forms of plant propagules such as bulbs, tubers, corms and rhizomes and explants used for plant vegetative propagation such as vegetative cuttings, scions, or *in vitro* cultured tissues as leaf and stem explants, anthers, *calli*, cell cultures, microspores, ovules, protoplasts, etc., are also used. Gametes, usually inside the inflorescences, are also targeted to mutagenic treatments (immersion of spikes, tassels, etc. into mutagenic solutions, **see Chapter 14**).

3.2.2. Types of Treatment

Mutagenic treatments are usually performed on plant tissues and result in multiple different mutations induced in a large number of cells, most of which normally do not develop into new plants. *In vitro* culture methods provide an exception in the sense that, for species where regeneration protocols are established, any mutated cell has the potential to regenerate into a new plant and thereby transmit its mutations to the next, sexual or vegetative, generation.

Box 12.2: Precautions for (and inactivation of) alkylating, arylating and acylating compounds

Precautions for "Carcinogens": Carcinogens that are alkylating, arylating or acylating agents per se can be destroyed by reaction with appropriate nucleophiles, such as water, hydroxyl ions, ammonia, thiols and thiosulphate. The reactivity of various alkylating agents varies greatly ... and is influenced by solubility of the agent in the reaction medium. To facilitate the complete reaction, it is suggested that the agents be dissolved in ethanol or similar solvents. ... No method should be applied ... until it has been thoroughly tested for its effectiveness and safety on material to be inactivated. For example, in case of destruction of alkylating agents, it is possible to detect residual compounds by reaction with 4(4-nitrobenzyl)-pyridine.

1. Oxidation by potassium permanganate in sulphuric acid ($KMnO_4$ in H_2SO_4). The products of the reaction have not been determined. Degradation efficiency was >99.5%.

2. Reaction with sulphamic acid in hydrochloric acid solution (HCl). The strong hydrochloric acid causes displacement of the nitroso group. The nitrosyl chloride formed reacts with the sulphamic acid to form nitrogen and H_2SO_4. This reaction prevents any reformation of the nitrosamide. The products of the reaction are the corresponding amides produced by simple removal of the nitroso group. Degradation efficiency was >99.5%.

3. Reaction with iron filings in HCl solution. The strong HCl causes displacement of the nitroso group. The nitrosyl chloride formed is reduced by the iron filings in the acid to ammonia. This reaction prevents any reformation of the nitrosamide. The products of the reaction are the corresponding amides produced by simple removal of the nitroso group except for N-methyl-N′-nitro-N-nitrosoguanidine and N-ethyl-N′-nitro-N-nitrosoguanidine, where reductive removal of the nitro group causes the major products to be methylguanidine and ethylguanidine, respectively. Degradation efficiency was >99%.

4. Reaction with sodium bicarbonate solution ($NaHCO_3$). This weak base causes a slow, base-mediated decomposition. The rate of reaction is sufficiently slow so that any diazoalkanes that are formed react with the solvent before escaping from the solution. The products of the reaction have not been definitely identified. Degradation efficiency was >99.99% for N-methyl-N-nitrosourea, N-ethyl-N-nitrosourea, N-methyl-N-nitrosourethane and N-ethyl-N-nitrosourethane. The method is not suitable for N-methyl-N′-nitro-N-nitrosoguanidine, N-ethyl-N′-nitro-N-nitrosoguanidine, or N-methyl-N-nitroso-p-toluenesulphonamide.

5. Reaction with $NaHCO_3$ solution, then nickel-aluminum (Ni-Al) alloy and sodium carbonate (Na_2CO_3) solution, then potassium hydroxide (KOH) solution. The slow increase in pH of the solution produced by sequential addition of the bases causes a slow degradation of the nitrosamide. The degradation rate is sufficiently slow so that any diazoalkanes that are formed have time to react with the solvent before escaping from the solution. Degradation efficiency is >99.9%.

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Nitrosamides. Lunn, G. and Sansone, E.B. 1994. Destruction of Hazardous Chemicals in the Laboratory. New York, NY: John Wiley & Sons, Inc., p. 279.

Table 12.4: Half-life of some alkylating agents in water solution at different temperatures and pH [Unit: $T_{0.5}$(h)]

Mutagens	Temp (°C)	pH6	pH7	pH8
MNU	20 (22)	24	(2.3)	
ENU	20(22)	31	(2.4)	
EMS	20 (25)		93.2 (7.8)	
MNNG	22			2.5
ENNG	22			7.0

One of the practical issues that concerns researchers and breeders is the optimization of the mutagenic treatments of the generative cells that will transmit mutations to the next (M_2) generation *via* sexual reproduction while lowering the injury of other tissues which will decrease plant survival or result in higher sterility in the M_1 generation.

During chemical mutagenic treatments, time (usually a few hours) is needed for the mutagen to reach the apical and/or axillary meristems in seed embryos, propagules, buds, etc., which are protected, respectively, by the seed testa and cotyledons and/or by primordial and adult leaves. In addition, during the mutagenic treatments the chemical mutagens undergo spontaneous degradation (**Table 12.4**) and part of the reactive chemical species will be lost in reactions (e.g. alkylation reactions) with the contents of tissues and

Table 12.5: Examples of mutagenic treatments with the most commonly used alkylating agents

Alkylating agent	Plant species	Plant material	Concentration (mM)	Exposure	References
MNU (MW 103.08)	*Begonia*	Leaf explants (*in vitro*)	0.2–10	1 h	Bouman and De Klerk (2001) Theor Appl Genet, 102: 111–117
	Lathyrus sativus	Pre-soaked seeds (12 h)	0.5–1.4	3 h	Rybinski (2003) Lathyrus Lathyrism Newsletter, 3: 27–31
	Lens culinaris	Seeds	0.49–3.88	6 h	Sharma and Sharma (1986) Theor Appl Genet, 71: 820–825
	Nicotiana glauca	Pre-soaked seeds (16 h)	1	2 h	Marcotrigiano and Hackett (2000) Annals of Botany 86: 293–298
	Oryza sativa	Panicles	1	45 min	Suzuki *et al.* (2008) Mol Genet Genomics, 279: 213–223
	Pisum sativum	Seedlings	1	1-4 h	Pereira and Leitão (2010) Euphytica 171: 345–354
ENU (MW 117.11)	*Brassica oleracea*				
	var. botrytis	Pieces of curd (*in vitro*)	0.3	Days	Deane *et al.* (1995) Euphytica, 85: 329–334
	Nicotiana plumbaginilfolia	Protoplasts	0.1	Days	Rey *et al.* (1990) Plant Cell Reports, 9 (5): 241–244
	Phaseolus vulgaris	Seeds	1.5–6.2	8 h	Svetleva (2004) J. Central European Agriculture, 5 (2): 85–90
	Pisum sativum	Seedlings	5	1–4 h	Pereira and Leitão (2010) Euphytica 171: 345–354
	Zea mays	Callus	1–30	4–8 h	Moustafa *et al.* (1989) Plant Cell and Tissue and Organ Culture, 17: 121–132
EMS (MW: 124.16)	*Glycine max*	Embryogenic cultures	1–30	4 h	Hofmann *et al.* (2004) Biologia Plantarum, 48 (2): 173–177
	Glycine max	Seeds	18	24 h	Wilcox *et al.* (2000) Crop Sci. 40: 1601–1605
	Helianthus annus	Pre-soaked seeds (4h)	80	5–12h	Nehnevajova *et al.* (2007) International Journal of Phytoremediation, 9: 149–165
	Oryza sativa	Panicles	94.2	(injected)	Lee *et al.* (2003) Genetics and Genomics, 22: 218–223
	Phaseolus vulgaris	Seeds	6.2–25	8 h	Svetleva (2004) J. Central European Agriculture, 5 (2): 85–90
	Trigonella foenum-graecum	Pre-soaked seeds (4h)	10–300	2–24 h	Saikat *et al.* (2008) Euphytica, 160: 249–258

cells other than the target meristematic cells, which will be reached some hours later by a depleted mutagenic solution.

To overcome this problem, the renewal – at least partial – of the mutagenic solution during the treatments has been suggested, which implies the repeated manipulation of the mutagen and the need for additional mutagenic solution. Another option is to pre-soak seeds (or other type of plant material) in water or buffer for some hours prior to mutagenic treatment. This allows the mutagen to diffuse more rapidly to the tissues of interest (meristems). Depending on the species and the experimental design diverse times of pre-soaking, from shorter periods of 4–5 hours to longer periods of 12–16 hours, may be used (**Table 12.5**). A third option is to pre-germinate seeds and to treat seedlings (**Table 12.5**). This procedure facilitates the access of the mutagens to the apical and axillary meristems and reduces the time of

exposure to 1–2 hours. This has advantages in providing almost full survival and fertility among the M_1 plants and very high mutation rates in the M_2 generation. A range of other tissues can be pre-soaked or pre-germinated for use *in vivo* and *in vitro* mutagenesis (bulbs, corms, tubers, etc.).

In certain specific circumstances, other types of treatment may be used in chemical mutagenesis, including dipping inflorescences such as spikes or tassels in mutagenic solutions, or injection of mutagenic solutions in stems or culms, etc. There are also some reports of *in vitro* treatments, in which plant tissues are cultivated in the continuous presence of low concentrations of mutagenic agents (**Table 12.5**).

3.2.3. Concentration of Mutagens, pH and Exposure Time

It is advisable that the concentrations of the mutagens to be used is established and reported in molar units. Molar units refer to the number of molecules of the mutagen in the solution and facilitates the comparison of the biological effects of different mutagenic agents on an equimolar basis. In many earlier works mutagen concentrations are reported as percentage units, (v/v) or (w/v).

Some general conclusions can be reached by comparing the most used alkylating agents EMS, ENU and MNU. In acute treatments, EMS is frequently used at ranges of 10–100s milli-molar concentrations (e.g. 10–100 mM and over); ENU is generally used at concentrations an order of magnitude lower (e.g. about 5–6 mM) while MNU is usually used at much lower concentrations (e.g. 0.2–1.0 mM). The most important limiting factor regarding the mutagenic concentration is the toxicity of the compound, which rapidly increases with concentration and shows a clear negative effect on the survival and fertility of the M_1 progeny.

When used as a solid substance, the alkylating agents need first to be dissolved in appropriate solvents such as ethanol or DMSO and then added to buffers or water. The pH of the buffers is usually neutral or slightly acid (pH 6–7), this helps to minimize degradation of the mutagens (**Box 12.2**). Commonly used buffers include the phosphate buffers, in particular the Sorensen's phosphate buffer, at pH 6.8–7.0. Distilled water is slightly acidic and is also commonly used.

Exposure time varies substantially depending on the type of plant material and concentration, but usually ranges from 1 to 6–12 hours.

3.3. Handling M_1 and M_2 Generations

As for physical mutagenic agents, the level of injuries induced by the alkylating agents in M_1 plants can be assessed as germination and/or field emergence rates and parameters such as survival–percentage of plants that attain the adult phase; sterility–percentage of adult plants that do not produce progeny; plant morphoses, chlorophyll chimeras, plant height, number of inflorescences, number of fruits, fertile branches, fertile nodes, number of seeds, etc. (**see Chapter 14**). Several parameters can be conceptualized and analysed in different ways, for example: sterility can be assessed establishing multiple levels of expression from less sterile plants to totally sterile plants.

Much work has been dedicated to correlating injuries to the M_1 plants with mutagenic rates in the M_2 progeny. This has been done in order to establish predictive rules in generating the required mutant variation in the subsequent M_2 generation. Lethal dose thresholds have also been investigated. It became commonly accepted that doses inducing 25 to 50% lethality (LD_{25}–LD_{50}) among the M_1 plants will result in the highest mutations rates. Most of these parameters were developed from treatments of dry seeds which required relatively long treatment times, and this resulted in relatively high lethal injury to embryonic tissues. However, the use of protocols that minimize the injury to M_1 plants (e.g. pre-soaking seeds or carrying out short mutagenic treatments on germinated tissues) allow very high mutation rates in the M_2 generation to be reached while significantly reducing the lethality among the M_1 plants.

The normal, general mutation breeding methods apply to chemical mutagenesis. The pedigree method where M_1 plants are individually harvested and individually sown as M_2 families and the bulk method where all M_1 plants are bulk harvested and the bulked M_2 progeny is sown are commonly used. These two main breeding methods contrast in the way mutation rates are calculated: 1) Pedigree method – as percentage of M_2 families with mutations and 2) bulk method – as percentage of mutated M_2 plants. The first method however, permits a better correlation to be established between the M_1 and M_2 generations, as well as providing a better comparison between biological effects of different mutagens.

In a broad sense, and often in practise, an M_2 family is taken to be the progeny of an M_1 plant. However, this concept can be further constrained and an M_2 family

can be formed from the progeny of a plant branch, individual spike, etc.

4. Other Chemical Mutagens

Paraphrasing the title of an article published by Ferguson and Denny (1995) it can be said that multiple groups of chemical compounds can be classed as an "underutilized resource" in plant chemical mutagenesis. Some of the promising compounds are discussed below.

4.1. Nitrous Acid and Nitric Oxide

4.1.1. Properties

The mutagenic effect of nitrous acid (HNO_2) in virus, bacteria, fungi and yeast was documented more than five decades ago. More recently nitric oxide (NO) was found to exert, through similar pathways, similar effects on biological systems. Both compounds induce two major types of mutations: transitions and DNA inter-strand cross-links.

Although hydrolytic deamination of the bases in DNA occurs spontaneously, nitrous acid and nitric oxide increase deamination rate, in particular of guanine. Both compounds induce nitrosamine deamination of adenine to hypoxantine (Hx), of cytosine to uracil and of guanine to xanthine (Xan) and oxanine (Oxa) at a molar ratio of 3:1 (**Figure 12.6**).

Hypoxantine pairs with cytosine lead to AT→GC transitions, while uracil pairs with adenine induce CG→TA transitions. Xanthine and oxanine can pair with cytosine or with thymine, in the latter case leading to GC→AT transitions.

Inter-strand cross-links are formed preferentially in 5′CG sequences where the two guanines on opposite strands remain covalently linked through a shared exocyclic amino group (**Figure 12.7**).

Uracil and hypoxantine are repaired by the BER pathways, whereas an alternative repair pathway is deployed for hypoxanthine involving endonuclease V (recently identified in *E. coli*). Oxanthine seems to be repaired less efficiently than xanthine, *via* BER or NER pathways. The fate of the nitrous acid and nitric oxide-induced DNA inter-strand cross-links as well as the consequences on the ability of oxanine to form cross-link adducts with aminoacids, polyamines and proteins are currently very poorly understood.

In plants, nitric oxide is assumed to play an important role in plant growth and development and to be a ubiquitous signal involved in the induction of cell death and of defence genes, and in the interaction with reactive oxygen species during defence against pathogens or in response to the plant hormone ABA. Bearing in mind that nitric oxide over-production in chronically inflamed tissues has been implicated in carcinogenesis and that NO releasing compounds are potent growth inhibitors of cancer cell lines and prevent colon and pancreatic cancer in animal models, the study of nitrous acid and nitric oxide effects in biological systems is expected to expand.

4.1.2. Nitrous Acid and Nitric Oxide in Plant Mutagenesis

Although, nitrous acid mutagenesis is used for genetic improvement of virus, fungi and bacteria for multiple biotechnological purposes, its exploitation in plant genetic improvement has been lacking. Since nitrous acid is quite unstable, the mutagenic treatments with nitrous acid need to be quick, e.g. not longer than a few minutes, which makes the treatment of dry seeds ineffective. Young seedlings and especially *in vitro* cultured plant cells, tissues and other vegetative explants offer more potential as targets for nitrous acid mutagenesis. The low pH at which nitrous acid needs to be maintained is an additional stress factor that needs to be considered in any nitrous acid treatment of plant materials.

Protocols for nitrous acid (HNO_2) mutagenic treatments: nitrous acid needs to be prepared fresh at low temperatures (0°C) before each treatment. Usually potassium or sodium nitrites are solubilized in acetate buffer at pH 4.0–5.5. Alternatively, identical volumes of solutions with equi-molar concentrations of sulphuric acid and barium nitrite are mixed and the barium sulphate removed by centrifugation. This last solution has a very low pH (1.5). Mutagenic treatments are usually performed with 0.02 to 0.1 M solutions.

4.2. Base Analogues and Related Compounds

4.2.1. Types and Effects

Under specific conditions some base analogues (**Figure 12.8**), and their ribosides and deoxyribosides, can be more mutagenic to specific organisms than the alkylating agents. The analogue of thymine 5-bromo-uracil

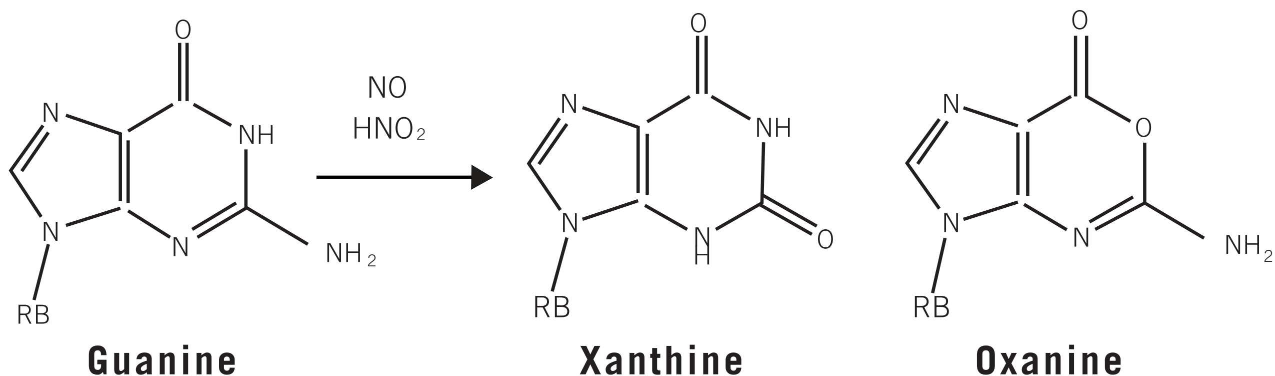


Figure 12.6 Nitrous acid and nitric oxide induce the nitrosamine deamination of guanine to xanthine and oxanine.

(BU) – and its deoxyriboside 5-bromo-2′-deoxyuridine (BUdR) – can incorporate into DNA and induce GC→AT and AT→GC transitions, and AT→TA and CG→AT transversions, as well as small indels that cause frameshift mutations, both *in vivo* and *in vitro*.

The ability of BUdR to incorporate into newly synthesized DNA strands is exploited in sister chromatid exchange analyses, and the anti-proliferative and radiosensitizing properties of this compound are being tested for anti-neoplastic treatments.

Other base analogues such as 2-aminopurine (2AP), 2,6-diaminopurine(2,6DAP), 6-N-hydroxylaminopurine (HAP) and 2-amino-6N-hydroxylaminopurine (AHAP) can function as analogues of adenine or guanine and can be incorporated into DNA where they induce high rates of C:G to T:A and T:A to C:G transitions and frameshift mutations. 2AP and 2,6DAP are very effective mutagens to phages and bacteria but weak mutagens to eukaryotic cells and weak carcinogens in mammalian systems. In contrast, HAP and AHAP are strongly mutagenic to eukaryotic cells and relatively carcinogenic. The clastogenic effect of base analogues in plants, including related compounds such as the alkylated oxypurines: 8-ethoxycaffeine and 1,3,7,9-tetramethyluric acid, was established over three decades ago almost simultaneously with the determination of the ability of some of these compounds to induce visible mutations in plants. Nevertheless, base analogues have not been tested intensively for the induction of mutants of interest in plants, and in this regard, the release of the malt-

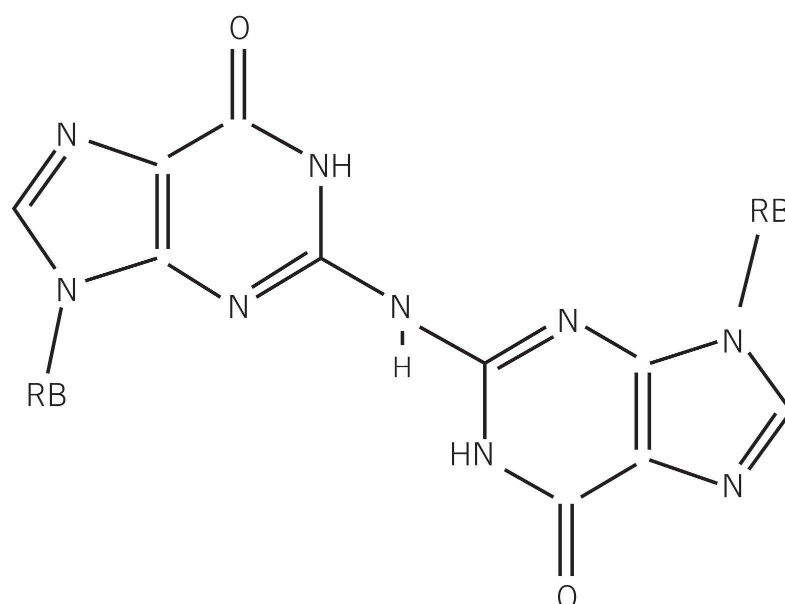


Figure 12.7 Covalent linkage of two guanines, on opposite strands, induced by nitrous acid and nitric oxide.

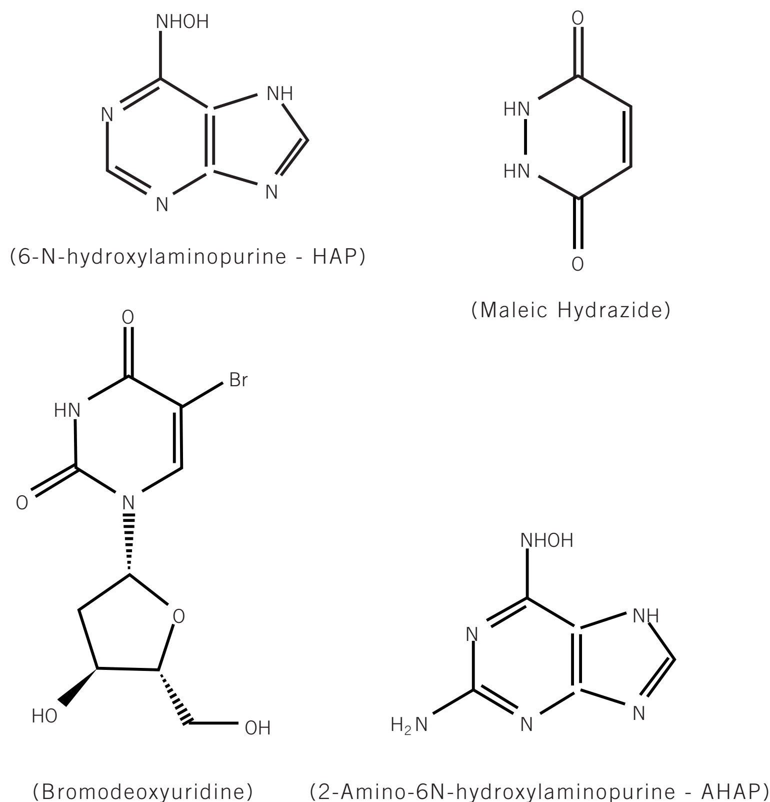


Figure 12.8 Molecular structures of several base analogues.

ing barley commercial variety "Fuji Nijo II" induced by BUdR + gamma rays treatment in Japan is exceptional.

Maleic hydrazide (1,2-dihydro-3,6-pyridazinedione, MH) is a structural isomer of uracil with plant growth regulator properties and is commonly used as a herbicide and sprout inhibitor. The genotoxic effects of this base analogue, particularly in plants, are rather intriguing and deserve further enlightenment. MH shows low mutagenic activity in bacteria, fungi and animal cells with seemingly no carcinogenic effects. However, this compound exhibits high mutagenic, clastogenic and recombinational activity in plants, frequently stronger than that of the most powerful alkylating agents (Gichner, 2003). In an experiment carried out simultaneously in two different laboratories the results of the comparative assessment of the genotoxicity of MH *versus* methyl methanesulphonate (MMS) in onion (*Allium cepa*) confirmed the much stronger clastogenic effect of MH: 24.0 and 46.4% cells with chromosome aberrations for concentrations of 5 mg/l and 10 mg/l, respectively, *versus* 19.1% aberrant cells for 10mg/l MMS (Rank *et al.,* 2002).

MH effects revealed by comet assay were contrary to the expected, since no DNA damage was observed (Gichner, 2003). However, Juchimiuk *et al.* (2006) also used the comet assay and registered extensive DNA damage both in *N. tabacum* and human leucocytes when MH treatments were performed on previously isolated cell nuclei. The comparison of the contradictory results of these experiments raises the question of the role of *in vivo* DNA repair mechanisms in the mutagenic effect of MH. Intriguing results were also observed when MH was used in combined mutagenic treatments. As expected, a synergistic effect was observed when MH was combined with X-rays in inducting somatic mutations in *Trandescantia* stamen hairs; but, conversely, an antagonistic mutagenic effect was registered when MH was combined with EMS.

4.2.2. Base Analogues and Plant Mutation Breeding

Apart from a very few exceptions, and despite the well documented genotoxic effects of MH in plants, reports on the use of this compound in plant mutation breeding

are lacking. The relative low toxicity and carcinogenicity of most of the base analogues, which are relatively inert and non-volatile compounds, are properties expected to encourage the utilization of base analogues in plant mutation breeding. MH and other base analogues have been used as water or buffer (e.g. Tris HCl) solutions at 5 to 10 mg/l.

4.3. Antibiotics

4.3.1. Types and Effects

Antibiotics are defined functionally on the basis of their anti-microbial activity. This group of compounds includes a multitude of natural and synthetically synthesized substances which, according to their molecular structure, fall into very different classes of compounds. Some antibiotics (**Figure 12.9**), such as streptozotocin, mitomycin C or azaserine, can also be included in the group of alkylating compounds.

Streptozotocin (STZ) is a naturally synthesized broad spectrum antibiotic and a potent mutagen and carcinogen used as a diabetogenic and anti-neoplastic agent. DNA-specific sequence analyses showed that over 98% of the STZ-induced mutations were G:C to A:T transitions with a few A:T to G:C transitions. However STZ also produces DNA strand breaks, alkali-labile sites, unscheduled DNA synthesis, DNA adducts, chromosomal aberrations, micro-nuclei, sister chromatid exchanges and cell death. Although the ability of streptozotocin to induce visible mutations in plants, in particular chlorophyll mutations, was reported four decades ago this mutagen has not been taken up in plant breeding experiments.

Mitomycin C (MMC) is an antineoplastic antibiotic isolated from *Streptomyces caespitosus* that inhibits DNA, RNA and protein synthesis and induces apoptosis in mammalian cells, and intra-chromosomal recombination in plant somatic cells. MMC is a bi-functional alkylating agent that reacts with guanine residues to form DNA inter-strand cross-links at the 5-CG-3 sequences and six types of guanine adducts, four of them arising from the direct alkylation of DNA by MMC and two other resulting from the previous formation of 2,7-diaminomitosene (2,7-DAM), which then alkylates DNA (Paloma *et al.,* 2001).

Azaserine is a naturally occurring serine derivative with antineoplastic properties that functions as a purine antagonist and a glutamine amidotransferase inhibitor. Azaserine spontaneously decomposes to diazoacetate which carboxymethylates DNA, forming O^6CmG, N^7CmG and N^3CmA as major products and O^6meG and N^7 and N^3 methylpurines as minor products.

The fluoroquinolone ciprofloxacin is a bacterial gyrase inhibitor that causes DNA DSBs and induces a wide pattern of mutations including different kinds of base pair substitutions and 3 or 6 base pair insertions/deletions that result in frameshift mutations in bacteria. At high concentrations ciprofloxacin inhibits the eukaryotic topoisomerase-II and induces genotoxic effects in mammalian cells in *in vitro* studies, whereas no genotoxic or carcinogenic effects were observed when used in *in vivo* tests with rodents.

Some antibiotics are radiomimetic and induce predominantly SSBs and DSBs in DNA. A major representative group of these antibiotics are the bleomycins, a group of natural glycopeptides produced by *Streptomyces verticillus*. It has potent antitumor activity, which is commonly assigned to its strong induction of DNA breakage (Ferguson and Denny, 1995).

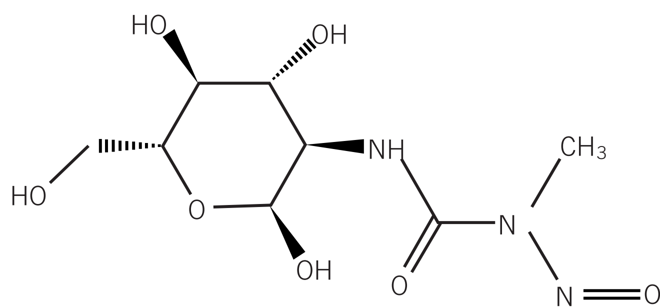
Many other antibiotics, such as fumagillin, amoxicillin and amoxicillin-related antibiotics, such as ampicillin, are also mutagenic to bacteria and mammalian cells, while others such as actinomycin D are potent apoptotic agents.

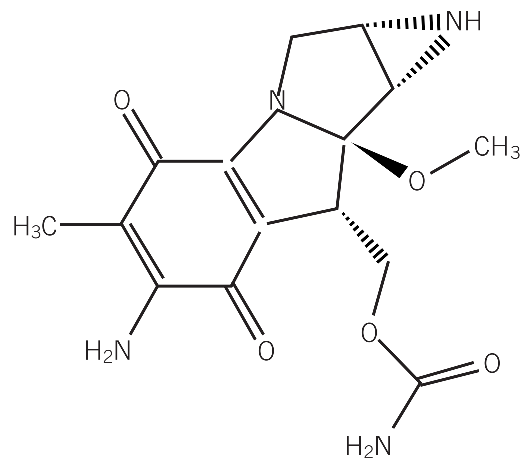
4.3.2. Antibiotic Mutagenicity Assays in Plants

The assessment of genotoxic effects of some antibiotics, in particular the study of the induced DNA lesions and respective repair pathways, has been carried out in plants, though at a much lower scale compared with the number of similar studies performed in bacteria and mammals.

The clastogenic properties of bleomycin have been determined in faba beans (*Vicia faba*), barley (*Hordeum vulgare*) and *Crepis capillaris* root cells. The formation and repair of bleomycin-induced DNA breaks in faba beans was assessed by neutral elution and by the comet assay. The very fast repair of bleomycin-induced strand breaks was first documented in *Arabidopsis* and more recently confirmed in barley (Georgieva and Stoilov, 2008).

Among the non-alkylating mutagenic compounds antibiotics have been the most extensively used in plant mutation breeding, with particular success in the induction of male sterile mutants in a number of plant species. Twenty-two cytoplasmic male sterile (CMS) and seven nuclear male sterile (NMS) mutants were selected in





The Joint FAO/IAEA Programme

Figure 12.9 Molecular structures of two antibiotic/alkylating agents.

sunflower (*Helianthus annus*) after treatments of seeds with mitomycin C and streptomycin. Streptomycin proved to be more effective in the induction of male sterility mutations as 18 of the CMS mutants were induced by this antibiotic. Six of these CMS lines have been released by the USDA-ARS and the North Dakota AES (Jan and Vick, 2006). The efficiency of streptomycin to induce male sterile mutants was also proved in sugar beet, sorghum and pearl millet. In this last species male sterile mutants were also induced with mitomycin C. Streptomycin, penicillin, rifampicin, erythromycin and tetracycline were also tested for inducting male sterile mutants in *Linum usitatissimum*.

Despite the very limited use of antibiotics in plant mutation breeding and the very few published studies on the genotoxicity of these compounds in plants, it is worth mentioning that among the new plant genomic tools stands the publicly available microarray data from the AtGenExpress initiative (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp). This includes the transcriptional response of 16-day old WT (Col) *Arabidopsis thaliana* seedlings to the genotoxic treatment with (1.5μg/ml) bleomycin and (22 μg/ml) mitomycin C for different (up to 24 hours) exposure times. Treatment conditions with antibiotics can vary substantially. Treatments with 50 to 200 μg/ml bleomycin, 5 to 500 μg/ml mitomycin C, 5 to 5000 μg/ml streptomycin and 1–5 mg/ml azaserine have been used.

4.4. Intercalating Agents and Topoisomerase Inhibitors and Poisons

4.4.1. Intercalating Agents

Intercalating agents can reversibly intercalate with double-stranded DNA, but they do not covalently interact with it. Classical intercalating compounds such as acridinium salts, ethidium bromide and propidium iodide are fused-ring aromatic molecules with positive charges on an attached side chain and/or on the ring system itself. Other compounds, such as DAPI, contain unfused aromatic systems with terminal basic functions and are classified as non-classical intercalators.

Initially used as disinfectants and anti-parasitic compounds, acridines and acridine derivatives constitute one of the biggest groups of the intercalating agents. These compounds have light absorbing properties and photo-enhanced cytotoxicity and mutagenicity and are exploited as dyes in biological and biochemical assays (e.g. acridine orange) and in clinics.

The most specific DNA lesion induced by the acridine compounds are frameshift mutations. The exploitation of these particular mutagenic properties allowed Francis Crick and co-authors to demonstrate the triplet nature of the genetic code using the acridine dye proflavine.

The genotoxic activity of acridines and related molecules differ from compound to compound. While simpler molecules, such as 9 amino-acridine and quinacrine, induce

±1 frameshift mutations in bacteriophages and bacteria and are weak clastogens and mutagens to mammalian cells, other acridines such as quinacrine, amsacrine and anthracyclines are severe clastogens and carcinogens.

Acridines bearing additional fused aromatic rings (benzacridines) show little activity as frameshift mutagens, but following metabolic activation interact covalently with DNA inducing predominantly base pair substitution mutations (Ferguson and Denny, 2007).

Nitroacridines have been shown to be mutagenic and clastogenic to mammalians. Some of these compounds, such as nitracrine and the 3-nitroacridine Entozon, exhibit a predominant induction of -2 frameshift mutations. Like the nitroacridines, the acridine mustards and aflatoxin B1 can induce frameshift mutations and base pair substitution.

Acridine mustards, in which an alkylating mustard is attached to an intercalating acridine chromophore, can be 100-fold more cytotoxic than the free mustard. Some acridine mustards such as ICR-191 and C20 are known to form adducts to the N^7 position of guanine, but analogues with longer linker chains, such as C5, can form adducts almost exclusively at the N^1 position of adenine.

The recombinogenic and mutagenic activities of some mutagens, e.g. bleomycin, can be enhanced and synergistic effects can be observed with pre-treatments with aminoacridines, nitroacridines or acridine mustards.

4.4.2. Topoisomerase Poisons

Topoisomerases play critical roles in primary DNA processes such as replication and recombination as well as in chromosome segregation, condensation and decondensation. While type I topoisomerases remove super-helical torsions in DNA generating temporary single breaks in one strand, type II topoisomerases alleviate DNA over-winds and resolve DNA knots and tangles by a similar mechanism but generate transient DSBs. In their function, topoisomerases establish the so called cleavage complexes, constituted by transient covalent attachment between the tyrosyl residues of their active site and the terminal DNA phosphates generated during the cleavage reaction. Compounds that stimulate the formation or increase the persistence of these topoisomerase-DNA cleavage complexes are referred to as “topoisomerase poisons”. Although there is some evidence for the involvement of topoisomerases in the induction of frameshift mutations, e.g. a functional T4 topoisomerase is required for 9-aminoacridine mutagenesis in T4 bacteriophage, the strong clastogenicity of several intercalating agents has been ascribed to their topoisomerase II poisoning properties.

Topoisomerase II poisons are strong cytoxic and/or clastogenic compounds, since stabilized cleavage complexes can inhibit DNA replication and cause DSBs. Multiple anti-cancer drugs such as etoposide and doxorubicin and antibiotic compounds such as ciprofloxacin and levofloxacin function as topoisomerase II poisons.

A second group of drugs that function by inhibiting the DNA binding and/or the catalytic cycle of topoisomerases are referred to as topoisomerase catalytic inhibitors. They do not induce DSBs and exhibit lower cytoxicity. Examples of eukaryotic (human) catalytic inhibitors are aclarubicin and merbarone; the former prevents the binding of topoisomerase II to DNA and the latter inhibits the DNA strands cleavage activity of this enzyme (e.g. McClendon and Osheroff, 2007).

Compounds that exhibit topoisomerase II poisoning activity can be found either among intercalating or non-intercalating agents. Genistein (a bioflavonoid), quinolones (e.g. CP-115,953), etoposide and teniposide are non-intercalating topoisomerase II poisons, while amsacrine, doxorubicin, mitoxantrone, proflavine and auramine are examples of intercalating topoisomerase II poisons (**Figure 12.10**). Quinones, which act as topoisomerase II poisons in the presence of cleavage complexes and as topoisomerase catalytic inhibitors when previously incubated with the enzyme in the absence of DNA, and benzene are other examples of strong topoisomerase poisons. Topoisomerase II catalytic inhibitors can also be classed as intercalating agents, such as 9-aminoacrine, chloroquine, tacrine and ethidium bromide or non-intercalating as merbarone.

Interestingly, topoisomerase II catalytical inhibitors can antagonize topo II poison activities. The attenuation of the DNA cleavage-enhancing properties of teniposide, amsacrine and etoposide by merbarone, or the inhibition of the etoposide induced micro-nuclei formation by chloroquine, A-74932, 9-aminoacridine and ethidium bromide, are examples of such antagonistic interaction.

Topoisomerase II does not become a genotoxic enzyme uniquely by the direct poisoning effect of chemical compounds. Poisoning effect on topoisomerase II

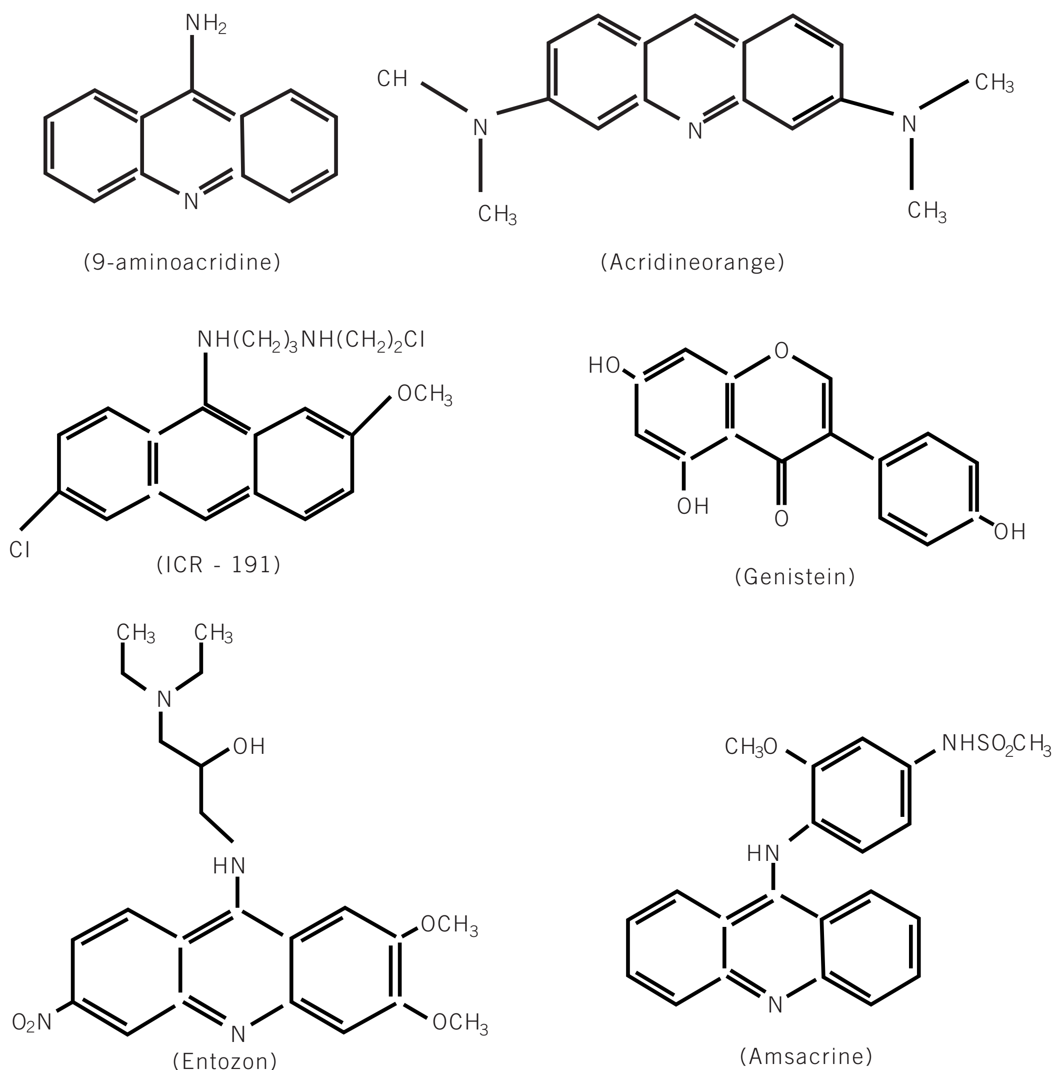


Figure 12.10 Molecular structure of several intercalating agents, topoisomerase catalytic inhibitors and topoisomerase poisons.

can be produced by double helix distortions caused by the presence of abasic sites generated by spontaneous hydrolysis, by DNA damaging agents or by base excision repair pathways (McClendon and Osheroff, 2007). In spite of the well demonstrated mutagenic activity of the intercalating agents and/or topoisomerase II catalytic inhibitors and topoisomerase II poisons in multiple prokaryotic and eukaryotic systems, and despite the well documented clastogenic and cytotoxic effects of acridines and their amino derivatives (Rank *et al.,* 2002), so far these compounds have been rarely tested in plants.

Intercalating agents and/or topoisomerase inhibitors and topoisomerase poisons have been used to induce mutants in bacteria, blue-green algae, *Chlamydomonas*, fish, animal and human cell lines, *Oenothera* chloroplasts, etc. However, to the best of our knowledge (except for a report of male sterile mutants induced by acriflavine and ethidium bromide in sugar beet) no other major publication has been produced reporting the use of this kind of compound for plant mutagenesis. The strong ability to induce frameshift mutations, DSBs and other types of mutational events, in some cases mediated by adduct formation, provides compelling reasons for more studies in plant systems. They might also become a new type of mutagen that can induce unique mutations for plant molecular biologists, e.g. for TILLING experiments, and for plant breeders.

Treatments with these mutagens are usually performed with low micro-molar solutions prepared from 5–10 mg/ml stock solutions in dimethylsulphoxide (DMSO), 10 mM HCl, 10mM KOH or in water. Optimal concentra-

tions and exposure times for plant mutation breeding purposes need to be determined. Although plant mutagenic treatments using these compounds can be performed on seeds, it is to be expected that compounds with mutagenic effects associated with topoisomerase poisoning or topoisomerase inhibition activities will be more effective on vegetatively growing tissues, particularly on seedlings and *in vivo* and *in vitro* explants.

5. The Resurgence of Chemical Mutagenesis and Practical Tips

5.1. Resurgence of Chemical Mutagenesis

The mutagenicity of alkylating agents was largely determined by I. Rapoport and co-workers, and readers able to read Russian can find an enormous amount of relatively old literature reporting a panoply of induced mutations in a wide range of biological subjects, including plants. However, the most important sources of literature on plant chemical mutagenesis are the discontinued *Mutation Breeding Newsletter* and its successor, the *Plant Breeding & Genetics Newsletter*, as well as the proceedings of meetings and other literature edited by the IAEA. Another good source of information on chemically induce mutants are the "newsletters" edited by International Societies for specific crops, e.g. pea and barley, as well as web pages and information published by germplasm centres and curators, e.g. John Innes Institute, UK.

In recent years, experimental mutagenesis has re-emerged as an important tool for plant reverse and forward genetics. Large mutagenized plant populations are produced and used to screen both for induced phenotypically expressed mutations and for mutational changes in specific genomic sequences using a procedure commonly called TILLING (targeting induced local lesions in genomes, see **Chapters 21 and 22**). Although physical mutagenic agents such as gamma radiation and fast neutrons and other mutagens such as sodium azide (NaN_3) have been used to produce mutant populations for TILLING analyses, the alkylating agents, in particular EMS but also MNU, are the most frequently utilized mutagens for that purpose. As a consequence there has been a resurgence of mainstream articles and references to plant chemical mutagenesis.

5.2. Practical Tips for Plant Mutagenesis Experiments

The guide lines below were developed for working with strong mutagens and carcinogens such as alkylating agents, but may be followed when using mutagens assumed to be less toxic, less mutagenic or less carcinogenic.

1. Alkylating agents are very toxic and very strong mutagens and carcinogens. This should always be kept in mind when handling these substances, health and safety should be the first consideration.
2. Handling chemical mutagens is relatively safe if safety rules are strictly observed. It is worth mentioning that handling chemical mutagens with excessive fear can be dangerous. Fear overcomes rational behaviour and the risk of accidents (usually minor) increases.
3. Lab coats and gloves are absolutely mandatory when handling chemical mutagens. Protective eye covers are also mandatory in all operations not performed behind the protection of a fume hood front glass or other protected cabinet.
4. Vials containing alkylating agents should always be opened in a fume hood or other specialized containment facility due the possibility of toxic gas formation, sometimes under pressure.
5. Gloves do not protect completely from mutagenic solutions and should never be in contact with the compound, either solid or in solution. Gloves should be changed frequently and disposed of in a specific designated bag.
6. The weighing of solid alkylating agents should be done as quickly as possible, but not in a rush. The required amount should be calculated. As soon as a quantity close to the needed amount is reached weighing must be stopped. The final concentration of the solution can be corrected adding the appropriate amount of water or buffer.
7. Weighing can be avoided using pre-weighed mutagens in sealed bottles. Solid mutagens such as MNU or ENU can be acquired in ISOPAC vials to which solvents (buffer, DMSO, ethanol) can be added *via* injection through a resealable cap.
8. Solutions are safer to handle than substances in a powder/solid form. Liquid spills are also much easier to locate than small powder particles. When

weighing an alkylating agent it is advisable to add some millilitres of the solvent (DMSO, ethanol) immediately after weighing out the material – this will prevent further dispersal of the powder and will retain any liberated gas. It is also advisable that any tool (e.g. spatula) that has been in contact with the mutagen is immersed immediately in a solvent.

9. All surfaces, over which mutagens are supposed to be manipulated (space around balances, fume hood, etc.), can be covered with filter paper fixed with tape or other means. At the end of the treatment the paper can be carefully removed trapping any spilled particles or drops and disposed of in a specific designated disposal bag.
10. After the mutagen is dissolved in an adequate amount of buffer or water, the solution can be poured over the plant material to be treated. Plant materials that can cause splashes (seeds, tubers, corms, etc.) should first be placed into the container used for treatment (e.g. a simple glass beaker). Alternatively, the plant material can be immersed in the final volume of water or buffer solution to which the mutagen dissolved in a solvent (DMSO, ethanol) can be added with careful stirring (e.g. with a glass rod). During the mutagenic treatment containers, even in a fume hood, should be covered, e.g. by parafilm.
11. After pre-soaking, if the remaining water or buffer is removed, the amount of liquid taken up by the plant materials should be taken into account if an exact calculation of the final concentration of the mutagen is required.
12. At the end of the treatment period the mutagenic solution should be decanted or pipetted out, or the treated material removed from the mutagenic solution (easier for cuttings, tubers, corms, etc.). In both cases treated plant material should be washed immediately with water to remove the remaining mutagenic solution. After this, three or more changes of water should be used for at least one hour in order to remove as much of the mutagen as possible.
13. Immersion of the plant material in 10% sodium thiosulphate solution for a few minutes can help to inactivate the mutagenic agent.
14. Mutagenized plant material to be carried forward for *in vitro* culture needs to be surface sterilized after the mutagenic treatment.
15. Workers engaged in sowing, planting, grafting or *in vitro* culture of mutagenized material should be fully informed about the material they are manipulating. Careful supervision of workers is required to ensure that they wear appropriate protective clothing and gloves and that mutagenized materials are not manipulated directly by hand (even when wearing gloves). Direct contact with the treated material should be avoided; forceps can be used when sowing and planting and additional protection is necessary when preparing scions to reduce the risk of contact with the mutagen still in the plant material.
16. Chemical mutagenic treatments should always be performed by, or under the directed supervision of a specially trained officer to avoid contaminations and/or non-declared and non-eliminated contaminations.
17. Treatments can be performed in specialized laboratories or in common laboratories. In the last case, access to treatment rooms should be restricted. Formation of toxic gases (e.g. diazoalkanes from nitroso compounds at high pH) and their transportation to other laboratories *via* fume hood conduits should be avoided completely. Is advisable to perform mutagenic treatments out of regular working time.
18. Mutagenic solutions, including waste waters, should be inactivated and glassware and metallic instruments (spatula, forceps, etc.) chemically decontaminated. Plastics, paper, gloves, etc. should be incinerated (**Box 12.2**). Most commonly used alkylating agents can be inactivated by adding sodium thiosulphate solution (10% final concentration) to the mutagenic solution and mutagenic waste liquids, and by slowly increasing the pH using a sodium hydroxide solution or sodium hydroxide pellets (to 1% final concentration) and kept at least overnight to decompose. This will assure the complete inactivation of the mutagens.

A solution of 10% sodium thiosulphate and 1% sodium hydroxide, should be used for decontamination of glassware and disposable labware. Information regarding inactivation and disposal of specific mutagens can be found at http://toxnet.nlm.nih.gov/.

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