Published in final edited form as:
Chem Res Toxicol. 2011 June 20; 24(6): 781–790. doi:10.1021/tx200064a.

Evolution of Research on the DNA Adduct Chemistry of *N*-Nitrosopyrrolidine and Related Aldehydes

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Abstract

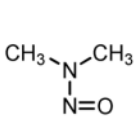
This perspective reviews our work on the identification of DNA adducts of *N*-nitrosopyrrolidine and some related aldehydes. The research began as a focused project to investigate mechanisms of cyclic nitrosamine carcinogenesis but expanded into other areas as aldehyde metabolites of NPYR were shown to have their own diverse DNA adduct chemistry. A total of 69 structurally distinct DNA adducts were identified and some of these, found in human tissues, have provided intriguing leads for investigating carcinogenesis mechanisms in humans due to exposure to both endogenous and exogenous agents.

Keywords

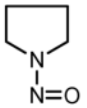
N-Nitrosopyrrolidine; NPYR; aldehydes; DNA adducts

Introduction

The remarkable discovery in 1956 by Magee and Barnes of the potent hepatocarcinogenicity of *N*-nitrosodimethylamine (NDMA, **1**), a simple water soluble compound with only 11 atoms, structurally quite different from most carcinogens recognized at that time, initiated a surge of interest in *N*-nitrosamines which had been known for nearly a century but ignored with respect to biological activity.[1] Energized by this discovery and the realization that *N*-nitrosamines could be readily formed in vivo or in foods by simple and rapid reactions of secondary or tertiary amines with nitrite, groups in Europe and the U.S. carried out extensive structure-carcinogenicity studies.[2–4] Ultimately, these researchers demonstrated the carcinogenicity of over 200 nitrosamines of varying structural types, and in 39 different species.[5] *N*-Nitrosamines were detected, sometimes in relatively high levels, in various foods (particularly cured and processed meats), beer, industrial cutting fluids, cosmetics, tobacco products, and human urine.[6–8] While *N*-nitrosamine levels in foods and beer have for the most part decreased substantially, significant contamination of tobacco products still exists today, and it is likely that cutting fluids, and perhaps other products still contain these carcinogens.[9–11]



NDMA (**1**)



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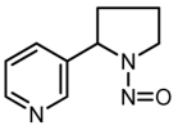

NNN (**3**)

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This perspective focuses on the DNA adduct chemistry of a representative cyclic nitrosamine, *N*-nitrosopyrrolidine (NPYR, **2**). Research groups led by Druckrey, Lijinsky, and others demonstrated that NPYR easily induced liver tumors in rats when administered in the drinking water.[4,12] While less carcinogenic than NDMA, NPYR nevertheless has considerable activity and is clearly more carcinogenic in the rat than some other well known carcinogens such as vinyl chloride, 2-acetylaminofluorene, and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ).[13] We became interested in NPYR because of its structural relationship to *N*′-nitrosonornicotine (NNN, **3**), a carcinogenic constituent of tobacco products that had been recently identified in unburned tobacco when this work began in the 1970s.[10,11,14] At that time, nothing was known about metabolism or DNA adduct formation of cyclic nitrosamines such as NPYR and NNN, although the pioneering studies of Magee and others had demonstrated that enzyme-catalyzed α-hydroxylation of NDMA led to the production of 7-methyl-Gua and O^6-methyl-Gua in DNA.[4] Based on this work and that of the Millers and others,[15] DNA adduct formation was considered to be crucial in the carcinogenic process, and that theory has withstood the test of time. There is no doubt today that carcinogen-DNA adducts lead to permanent mutations in critical cancer related genes that drive the carcinogenic process.[16,17] When DNA adduct formation from *N*-nitrosamines is blocked, so is cancer induction.[18]

Our studies on NPYR unexpectedly led us to an examination of DNA adduct formation by related aldehydes. In this perspective, we outline the evolution of this research. We focus on some of the more important results and some unanswered questions posed by these findings.

NPYR metabolism by α-hydroxylation

The first step was to characterize products of NPYR metabolism by the α-hydroxylation pathway. Using model compounds such as α-acetoxyNPYR (**4**), which is readily hydrolyzed to the critical but unstable intermediate α–hydroxyNPYR (**6**), at least partially via intermediate **5**, we demonstrated that the main product of enzymatic α-hydroxylation was 2-hydroxytetrahydrofuran (**13**), a cyclic lactol which is the predominant form of 4-hydroxybutyraldehyde (**9**) (Scheme 1).[19] This chemistry was completely consistent with known metabolic pathways of acyclic nitrosamines. Further studies showed that this important reaction was catalyzed by cytochrome P450 2E1 but poorly by P450 2A enzymes, and was observed in laboratory animals treated with NPYR as well as in human tissues.[20–22] 3-Hydroxybutanal (**10**), which is in equilibrium with the dimer paraldol (**14**), and crotonaldehyde (**11**) were also formed, presumably following a 1,2 proton shift in the carbocation derived from diazonium ion **8**.[23–26] It was the observation of crotonaldehyde (**11**) in these reactions that spurred our interest in DNA adduct formation by aldehydes. Equilibria exist among crotonaldehyde (**11**), 3-hydroxybutanal (**10**), paraldol (**14**), acetaldehyde, and related lactols, and all of these compounds are capable of reacting with dGuo and/or DNA. Thus, an initially conceived study of DNA adduct formation by NPYR expanded to include these related aldehydes, as well as acrolein and formaldehyde.

DNA adducts of NPYR and related aldehydes

Figure 1 summarizes the structures of the DNA adducts characterized in this work.[24–46] Adducts **15 – 32** are formed upon reactions of α-acetoxyNPYR with deoxyribonucleosides or DNA in vitro, or have been found in tissues of rats treated with NPYR. Adducts **33 – 47** are formed from related aldehydes. Altogether, including characterized stereoisomers, 51 adducts have been identified.

Adduct **15** is quantitatively the major DNA adduct produced in vivo in laboratory animals treated with NPYR[46]. Smaller amounts of adducts **16, 17, 20, 21, 24 – 29**, and **31** (in some cases after $NaBH_3CN$ treatment of the DNA) have also been observed. [31,32,43,44,46] Adducts

31 – 35 are produced in crotonaldehyde reactions, and in some cases acetaldehyde reactions, with DNA or deoxyribonucleosides, and **31** has also been observed in rodent and human tissues.[27,31,41,46–48] Adducts **36 – 40** are produced from reactions of acrolein with DNA, in some cases after reduction; **36** and **37** have been identified in human tissues.[28,39,47,49,50] Adduct **41** is produced in reactions of DNA with paraldol and crotonaldehyde and in the reaction of dGuo with aldoxane.[25,37] Adducts **42** and, after $NaBH_3CN$ treatment, **43**, are the major DNA modifications caused by acetaldehyde, while cross-link **44** is produced in DNA reactions with both acetaldehyde and crotonaldehyde.[36,42,45,51] Adduct **45** is formed in reactions of acetaldehyde and aldoxane with dGuo, and **46** in reactions of acetaldehyde with dGuo.[39] Adduct **47** is produced in reactions of acetaldehyde and formaldehyde with dGuo.[39]

Collectively, these results indicate the diversity of DNA damage that potentially could be caused by NPYR and related aldehydes. There is solid evidence based on inhalation studies for the carcinogenicity of acetaldehyde and formaldehyde; but they have never been compared directly to NPYR, and inhalation studies of NPYR have not been reported.[52,53] Based on comparison of TD_{50} values, NPYR is more carcinogenic than acetaldehyde or formaldehyde.[13] Studies on the carcinogenic properties of acrolein and crotonaldehyde are less convincing,[54,55] and no data are available for aldoxane, 3-hydroxybutanal, or paraldol. For the most part, we lack side by side comparisons of DNA damage in vivo by NPYR and these related aldehydes. The relatively higher carcinogenicity of NPYR compared, for example to crotonaldehyde,[55] is likely due in part to the generation of reactive intermediates, including aldehydes, intracellularly as opposed to extracellular delivery. The inherent mutagenicity of each adduct clearly will also affect the outcome.

Some DNA adducts: potential importance and future directions

In this section, we consider some research directions suggested by the diverse DNA adduct structures in Figure 1.

NPYR-DNA adducts—In a recent study, we used LC-ESI-MS/MS to analyze levels of adducts **15**, **21**, **25**, **28**, and **31** in hepatic DNA of NPYR-treated rats[46]. The goal was to compare the relative levels of these adducts using a reliable and validated technique. The results clearly showed that adduct **15** was by far the most prevalent, being formed in levels hundreds to thousands of times greater in hepatic DNA than the other adducts. The results obtained in that study, using LC-ESI-MS/MS, were quite consistent with those of previous studies that used a variety of different NPYR dosing protocols and adduct detection methods.[30–32,35,44,56,57] This result is logical based on the known DNA adduct chemistry of simple alkylating agents, in which N7 of Gua is commonly the most reactive site.[58] In the case of **15**, the 4-oxo substituent leads to cyclization at C8, consistent with the known lability of the C8 proton in Guo or DNA alkylated at the N7-position.[59,60] One direction clearly suggested by this result is analysis of human DNA for adduct **15**. If continuous, but probably low level, exposure to NPYR via both exogenous and endogenous mechanisms is occurring in humans, as we have suggested,[35] adduct **15** could be an excellent indicator of this exposure because of its unique structure. The excretion of adduct **15** in the urine of NPYR-treated rats has been demonstrated.[35] Furthermore, studies on the relative amounts of adduct **15** in dGuo vs. Guo, and DNA vs. RNA, demonstrate that Guo and RNA alkylation predominates, which also suggests unique approaches to biomarker development.[35] While detection of NPYR itself in urine has been reported in several studies, the significance of these findings is uncertain because very little NPYR is excreted unchanged in the urine of animals treated with this carcinogen. The major metabolite of NPYR is CO_2.[20]

What is the biological significance of adduct **15**? No data are available. However, spontaneous depurination of 7-alkyl-dGuo in DNA is well established.[58] This leads to an

abasic site, and to consequent G → T mutations.[61,62] However, these were not observed in the one reported study of in vivo mutational analysis of liver DNA in transgenic rats treated with NPYR.[63] In that study, the major mutations induced by NPYR occurred by far at AT rather than GC base pairs. These results strongly suggest that dAdo or dThd adducts such as **24** or the non-reduced precursors to **28** –**30**, are important in NPYR hepatocarcinogenesis. This is clearly a critical area for future research on NPYR carcinogenesis.

NNN-DNA adducts—Our original goal in studying NPYR metabolism and DNA adduct formation was to inform similar studies on NNN, which undergoes metabolic activation by 2′-hydroxylation and 5′-hydroxylation to intermediates **48** – **51** (Scheme 2). Extensive studies carried out to date clearly demonstrate DNA adduct formation in tissues of NNN-treated rats.[64,65] All adducts detected to date in these studies result from 2′-hydroxylation, proceeding through pyridyloxobutylation via intermediates **48** and **50**. The structures of these adducts, resulting from reactions of **50** and related intermediates with dGuo, dThd, and dCyt, as well as their formation and persistence in vivo, have been described.[66] DNA adduct formation from 5′-hydroxylation of NNN via intermediates **49** and **51** is formally more closely analogous to NPYR-DNA adduct chemistry, and the adducts identified to date from studies of the reaction of 5′-acetoxyNNN (**60**, Scheme 3) with deoxyribonucleosides or DNA have their counterparts in NPYR adduct chemistry. The structures of these adducts are summarized in Figure 2 and their modes of formation are outlined in Scheme 3.[67,68] Adducts resulting from reaction at the exocyclic amino groups of dGuo and dAdo, as well as the O^2-position of dThd have been observed. Thus, dGuo adducts **52** and **53** are analogous to NPYR adducts **20** and **21** (Figure 1), while adducts **54** and **55** are analogous to **22** and **23**. Similarly, dAdo adducts **56** and **57** are analogous to **24** and **25**, while dThd adduct **59** is analogous to **28**. Altogether, including diastereomers, **18** DNA adducts resulting from NNN 5′-hydroxylation have been characterized, but none of these adducts has yet been detected in vivo. Further studies are required to determine whether adducts resulting from 5′-hydroxylation of NNN are present in tissues of NNN-treated rats. For example, studies of NNN-DNA adduct formation by 2′-hydroxylation have consistently found higher levels in the respiratory than in the olfactory portions of the rat nasal mucosa, yet 5′-hydroxylation is efficiently catalyzed by P450 2A3 which is present in the olfactory mucosa, and malignant nasal tumors induced by NNN arise mainly in the olfactory portion of the nasal mucosa.[64] It will be important to determine whether DNA adducts resulting from 5′-hydroxylation of NNN, such as those shown in Figure 2, are present in relatively high levels in the rat olfactory mucosa after treatment with NNN.

We have so far found no evidence for a N7-C8 cyclic NNN adduct analogous to the major NPYR adduct **15**. Reactivity at N7 of Guo is generally favored by S_N2 as opposed to S_N1 character of the reactant, e.g. methylmethanesulfonate produces more 7-methyl-Guo relative to reaction at other sites than do *N*-alkyl-*N*-nitroso compounds which tend to react more at exocyclic centers such as O^6 or N^2.[58,69] This generalization however would not appear to explain the difference between intermediates 7 (Scheme 1) generated from NPYR and **51** (Scheme 2) from NNN. Positive charge in a carbocation derived from intermediate **51** would be destabilized by the pyridine ring, decreasing its S_N1 character.

Cyclic 1,N^2-dGuo adducts of crotonaldehyde and acrolein—Reaction of α-acetoxyNPYR (**4**) with dGuo led to the unexpected production of the cyclic 1,N^2-dGuo adducts **31**, which resulted from reaction of crotonaldehyde (**11**, Scheme 1) with dGuo.[27] This was the first structural characterization of a cyclic 1,N^2-dGuo adduct. Similar reactions were observed in DNA treated with either crotonaldehyde or acrolein, the latter producing adducts **36** and **37**.[28] These findings initiated a separate line of research by several groups that reached far beyond studies of NPYR metabolism and DNA adduct formation, focusing on DNA modification by α, β-unsaturated aldehydes including acrolein, crotonaldehyde, and

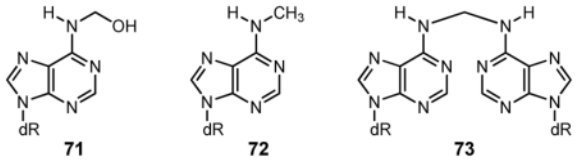
4-hydroxynonenal which themselves are exogenous toxicants or are produced endogenously in lipid peroxidation reactions. These studies, encompassing elegant approaches by a number of different groups, have explored the chemistry and biology of these DNA adducts, and have recently been reviewed.[70] The cyclic 1,N^2-dGuo adducts, commonly found in human and rodent DNA, are repaired by nucleotide excision repair. If unrepaired, they cause predominantly G→T transversions, although weakly. The adducts can undergo ring opening in DNA, leading to interstrand DNA cross-links or DNA-protein conjugates. The biological implications of these observations are still being explored.[70]

We have focused on the potential role of these adducts in tobacco carcinogenesis. The crotonaldehyde adducts **31** and the acrolein adducts **36** and **37** have been detected in human lung DNA, but their relationship to smoking in these studies was unclear.[41,49] In a recent study, we found no effect of smoking on levels of acrolein adducts **36** and **37** in human leukocyte DNA and suggested that efficient glutathione detoxification of acrolein may be protecting DNA from exposure to acrolein from cigarette smoke.[50] This topic requires further investigation in view of the finding that acrolein produces a mutation spectrum in the *p53* gene similar to that found in lung tumors from smokers, and therefore has been suggested as an important contributor to lung cancer in smokers.[71]

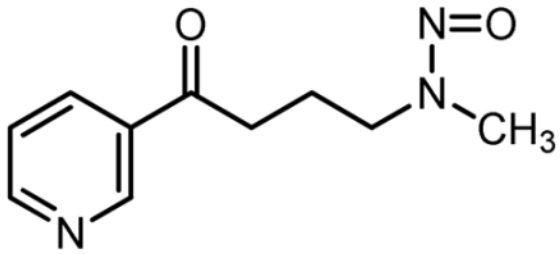
From crotonaldehyde to acetaldehyde—Further examination of the reactions of α-acetoxyNPYR and crotonaldehyde with DNA demonstrated that thermally unstable adducts were present. These were shown to be adducts **20**, **24**, and **26** from α-acetoxyNPYR, and **33** and **41** from crotonaldehyde.[24] Scheme 4 summarizes modes of formation of some of the more complex aldehyde adducts. Schiff base adduct diastereomers **33** (Figure 1) in particular were found in relative high concentrations in the crotonaldehyde reactions.[26] In contrast to adduct **31**, crotonaldehyde adducts **33** and **41** have yet to be identified in human DNA, but this is a worthy area for further study. The origins of these adducts from 3-hydroxybutanal (**10**, Scheme 4) suggested an investigation of acetaldehyde-DNA adducts, since **10** is simply the aldol condensation product of acetaldehyde. This led to the first identification of an acetaldehyde or crotonaldehyde induced cross-link in DNA, adduct **44** of Figure 1 and Scheme 4, along with the realization that the cyclic 1,N^2-dGuo adduct **31** of crotonaldehyde could also be formed from acetaldehyde by a stepwise mechanism (via **68** of Scheme 4), which yielded a different ratio of stereoisomers than the crotonaldehyde reaction.[36] Thus, adduct **31**, commonly found in human DNA and attributed to crotonaldehyde derived from lipid peroxidation, could also result from exposure to acetaldehyde, a quantitatively significant constituent of cigarette smoke (32 – 828 μg per cigarette) and the major metabolite of ethanol. A specific and major DNA adduct of acetaldehyde is N^2-ethylidene-dGuo (**42**). This adduct is fairly stable in DNA, but rapidly degrades at the nucleoside level.[36] Analysis of human hepatic and leukocyte DNA for this adduct can readily be accomplished by inclusion of $NaBH_3CN$ during the enzymatic hydrolysis of the DNA, thus converting **42** to the stable adduct N^2-ethyl-dGuo (**43**).[42,45] Some studies indicate that this adduct may be a good biomarker for alcohol consumption, but the results to date are incomplete and require confirmation.[72–74] Alcohol consumption is a known risk factor for head and neck cancer, particularly in *ALDH2* deficient individuals or in combination with smoking, and is also a commonly reported risk factor for breast cancer.[75,76] Acetaldehyde associated with alcoholic beverages is considered carcinogenic to humans by the International Agency for Research on Cancer.[75] Biomarker studies of adduct **42** promise to provide further insight into the etiology of cancers associated with alcohol consumption.

From acetaldehyde to formaldehyde—Our finding that the cyclic 1,N^2-dGuo adduct **31**, commonly attributed to crotonaldehyde, could also be produced from 2 molecules of acetaldehyde, led us to speculate that a similar sequence of reactions involving

formaldehyde and acetaldehyde could lead to adduct **36** (Figure 1), known to be a product of the reaction of acrolein with DNA. Therefore we investigated the reactions of formaldehyde plus acetaldehyde with dGuo.[39] Formaldehyde is a toxicologically significant molecule, considered to be a cause of nasal cancer in humans.[53] Our hypothesis was not supported by the results of that study, but our findings did demonstrate the ease with which formaldehyde-DNA adducts, including cross-links, were formed. These formaldehyde-DNA adducts had all been characterized previously by the elegant studies of Shapiro, Beland, and others.[77,78] The ease of formation of formaldehyde-DNA adducts in vitro encouraged us to examine their formation in vivo, which had not been previously reported. We developed an LC-ESI-MS/MS method for analysis of N^6-hydroxymethyl-dAdo (**71**), as N^6-methyl-dAdo (**72**) after $NaBH_3CN$ treatment of the DNA; the dAdo-dAdo cross-link adduct **73** was also analyzed.[79] We applied this method to the analysis of DNA from rats treated with NDMA(**1**). The results demonstrated that these formaldehyde DNA adducts were indeed formed in tissues of NDMA treated rats, in addition to the well known adducts 7-Me-dGuo and O^6-Me-dGuo.[79] The Swenberg group has recently used similar technology to quantify endogenous and exogenously induced DNA adducts (N^2-hydroxymethyl-dGuo) in tissues of rats and primates treated with [$^{13}CD_2$]formaldehyde.[80,81]



Since formaldehyde is present in cigarette smoke (2 – 76 ug per cigarette) and formaldehyde DNA adducts were also produced in tissues of rats treated with the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, **74**), as a consequence of metabolic α-hydroxylation, we extended our methodology to the analysis of leukocyte DNA from smokers.[82] These results demonstrated clear differences in levels of N^6-hydroxymethyl-dAdo (**71**) between smokers and non-smokers, suggesting a previously unrecognized role for formaldehyde in tobacco carcinogenesis. Further studies are planned to confirm and extend this observation.



NNK (**74**)

Summary

The studies described here were driven mainly by our interest in the structures of DNA adducts that could be formed from the carcinogenic cyclic nitrosamines NPYR and NNN. The ultimate characterization of 69 distinct DNA adducts was far beyond our initial aims and unexpectedly led to research by our group and others on DNA damage by aldehydes such as formaldehyde, acetaldehyde, acrolein, and crotonaldehyde, extending to such broad

topics as lipid peroxidation, alcohol consumption, and cancer etiology. These results demonstrate the critical role of chemistry in studies of carcinogenesis and toxicology.

The role of NPYR in human carcinogenesis is still unclear, but we now possess the tools to test its DNA damaging effects, for example by analysis of human tissues or urine for **15**. Mechanisms of NPYR carcinogenesis also require further study to elucidate the role of adduct formation at AT base pairs. There is no reason to doubt that NNN and NNK are among the principal causes of tobacco-induced cancer for reasons that have been discussed extensively elsewhere.[11] Still, there is a need to further understand human DNA damage by NNN specifically, perhaps by developing methodology for analysis of the adducts illustrated in Figure 2.

There is considerable human exposure to the aldehydes discussed here from both exogenous and endogenous sources and there is solid evidence, unique among all adducts in Figures 1 and 2, for the presence in human DNA of **31** (from crotonaldehyde and acetaldehyde), **36** and **37** (from acrolein), **42** and **43** (from acetaldehyde) and **71** (from formaldehyde). Reliable methods now exist for the quantitation of these adducts in human DNA, and one can envision using these methods to test their potential role in human cancer etiology through clinical and epidemiologic studies. Further studies on the repair and mutagenicity of some of these adducts are also needed. There are tantalizing clues in the literature regarding the involvement of aldehydes in human carcinogenesis, and the next phase of this research should explore these leads.

Acknowledgments

Professors Fung-Lung Chung of Georgetown University and Edward J. McIntee of the College of St. Benedict and St. John's University made substantial intellectual contributions to this research. We thank Peter W. Villalta for his dedicated involvement in mass spectrometry studies, and Bob Carlson for editorial assistance.

Grant Support. This research was supported by grants CA-85702 and CA-81301 from the National Cancer Institute. Mass spectrometry was supported in part by Cancer Center Support Grant CA-77598.

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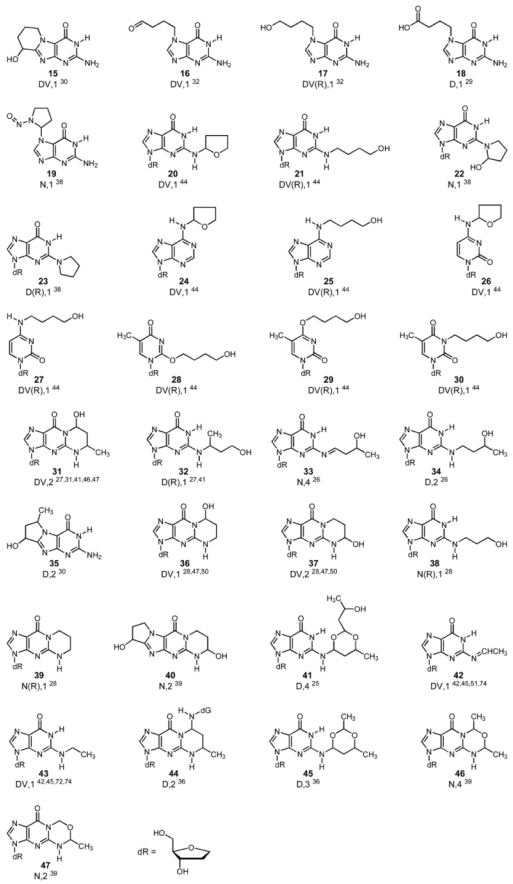


Figure 1.

DNA and deoxyribonucleoside adducts produced from NPYR and related aldehydes. Adducts **15** – **32** are formed upon reactions of α-acetoxyNPYR (4) with deoxyribonucleosides or DNA in vitro, or have been found in tissues of rats treated with NPYR. Adducts **33** – **47** are formed from related aldehydes. The symbols represent the identification of these adducts in animal or human DNA in vivo (DV), either as such or after $NaBH_3CN$ or $NaBH_4$ reduction (R), or in reactions with DNA in vitro (D), or in reactions with deoxyribonucleosides (N). (In each case, only the highest level of evidence is given, where DV is higher than D, and D is higher than N). The number following these letters represents the number of identified stereoisomers, and key references are given.

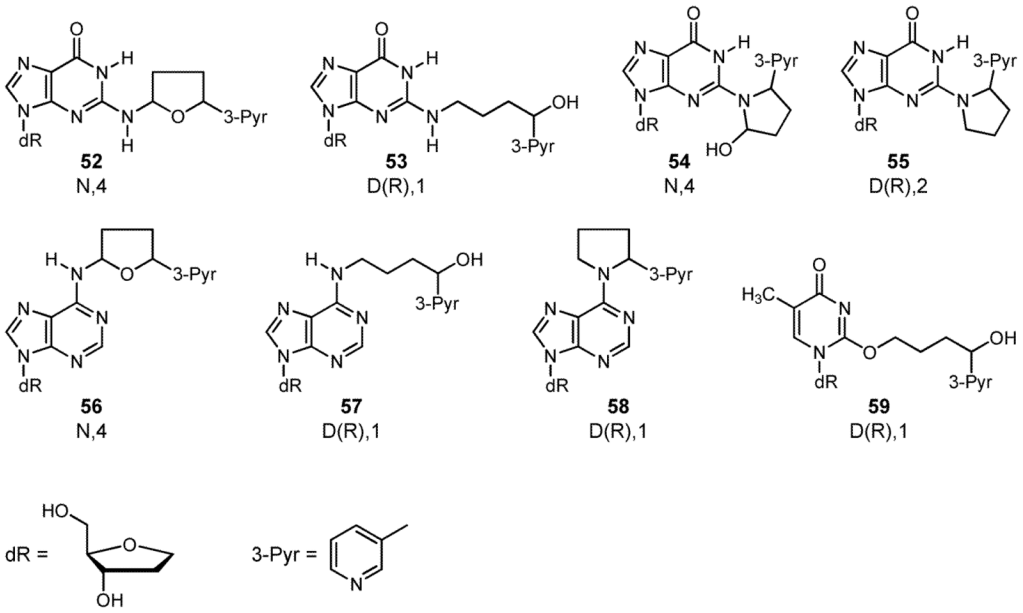
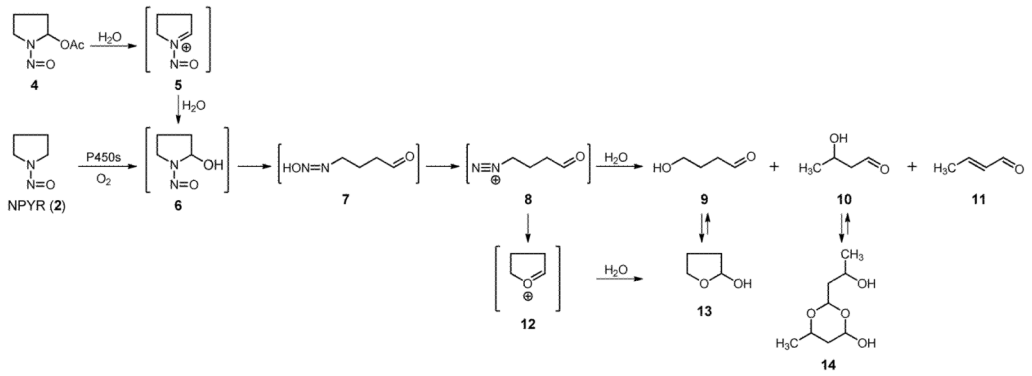
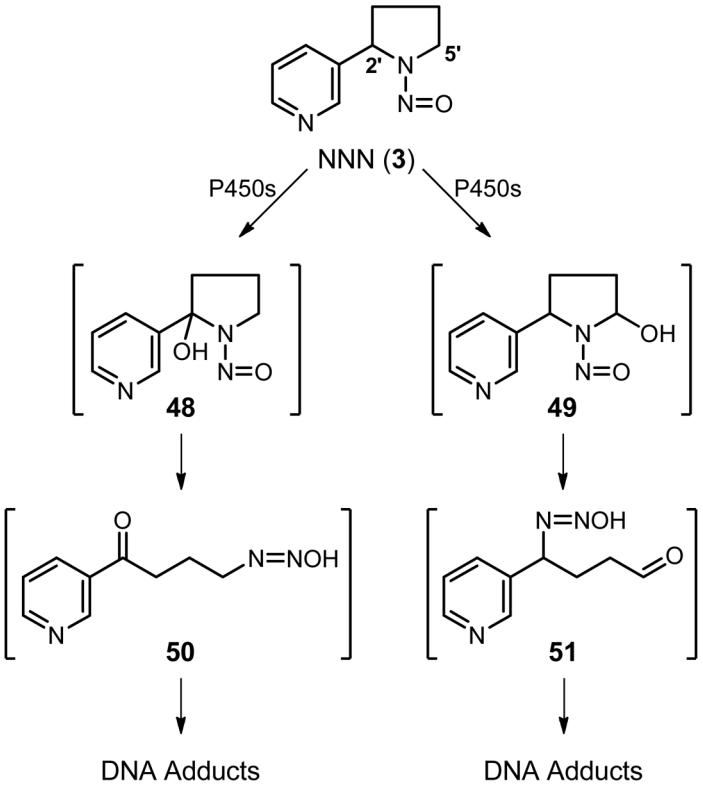


Figure 2.

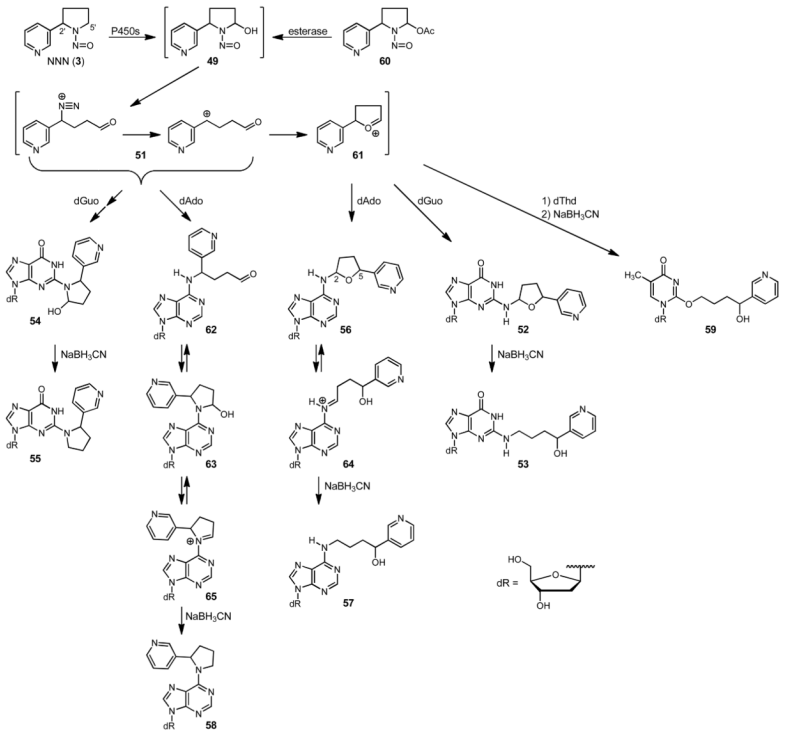
DNA and deoxyribonucleoside adducts produced upon 5′-hydroxylation of NNN (**3**).[67,68] The symbols represent the identification of these adducts in reactions with DNA in vitro (D), or in reactions with deoxyribonucleosides (N), in some cases with $NaBH_3CN$ treatment (R). The number following these letters represents the number of identified stereoisomers.



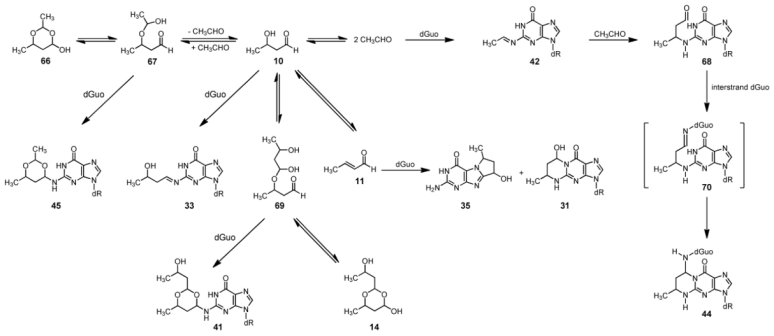
Scheme 1.
Some intermediates and products of NPYR metabolism by α-hydroxylation



Scheme 2.
Key intermediates produced in metabolic α-hydroxylation of NNN.



Scheme 3.
Adduct formation via 5′-hydroxylation of NNN



Scheme 4.

Pathways of formation of some of the more complex aldehyde adducts