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Review

Oxidative metabolism and genotoxic potential of major isoflavone phytoestrogens

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Abstract

The soy isoflavones daidzein, genistein and glycitein are extensively metabolized by rat liver microsomes to a variety of catechol metabolites. Hydroxylated metabolites of daidzein and genistein have also been demonstrated in incubations with human hepatic microsomes and in the urine of humans after ingestion of soy food. Although the microsomal metabolism of formononetin and biochanin A is dominated by demethylation to daidzein and genistein, respectively, catechols of the parent isoflavones and of the demethylation products are also formed. Thus, oxidative metabolism appears to be common among isoflavones and may have implications for their biological activities. As genistein but not daidzein exhibits clastogenic activity in cultured mammalian cells, the role of oxidative metabolism for the genotoxicity of isoflavones is of particular interest.

Keywords: Reviews; Isoflavone phytoestrogens

Contents

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1. Introduction

Phytoestrogens of the isoflavone family occur in numerous plants used for human and animal nutrition [1]. They are most abundant in soybeans, but are also present in appreciable concentrations in a variety of beans, sprouts and legumes. Animal feed such as clover or alfalfa are rich sources of isoflavones. Fig. 1 depicts the structures of the most common isoflavones. Their pattern varies between different plants; e.g. soybeans contain mostly daidzein (DAI) and genistein (GEN) together with a small amount of glycitein (GLY), whereas red clover is high in formononetin (FOR) and biochanin A (BCA).

In the plants, isoflavones are mostly present as 7-β-D-glycosides of glucose and 6″-*O*-malonylglucose. Upon ingestion, the aglycons are efficiently liberated from their glycoside forms and subjected, in part, to reductive metabolism by intestinal bacteria [2–4]. For example, typical bacterial metabolites of DAI are dihydro-DAI, *O*-desmethylangolensin and equol (Fig. 1). The bacterial metabolites and the isoflavones escaping bacterial biotransformation are absorbed in the intestine, where extensive glucuronidation occurs in the enterocytes prior to release into the blood and transport to the liver [5]. Conjugates with glucuronic acid and sulfate are excreted into urine and bile. Biliary metabolites are known to undergo enterohepatic circulation [5].

Very recently, it has been reported that isoflavones, in addition to reductive and conjugative metabolism, are prone to oxidative biotransformation in rats and also in humans [6,7]. The present paper reviews these studies and provides new data on the oxidative metabolism of several other isoflavone phytoestrogens. In view of the wide exposure to these compounds and the fact that structurally related estrogenic agents, e.g. the endogenous estrogen 17β-estradiol (E2) and the synthetic estrogen diethylstilbestrol (DES), are associated with cancer in humans and animals [8,9], the potential of phytoestrogens to cause genetic damage is of interest. Therefore, a brief account of the results of genotoxicity studies of isoflavones is given.

2. Methodology

The methods used for the generation, separation, and identification of isoflavone metabolites were described in detail in recent publications [6,7]. Briefly, isoflavones were incubated with microsomes prepared from the livers of aroclor-treated male Wistar rats or from human liver. The microsomal metabolites were extracted with ethylacetate and analyzed by HPLC with a diode array detector (DAD) and by HPLC–MS using a HP 1100 series quadrupole mass analyzer equipped with an atmospheric pressure ionization-electrospray ionization chamber. GC–MS was carried out on a Finnigan GCQ system connected to an ion trap mass detector. Urine samples of volunteers kept on a soy diet were solid-phase-extracted and the extracts hydrolyzed with β-glucuronidase/aryl sulfatase. Deconjugated

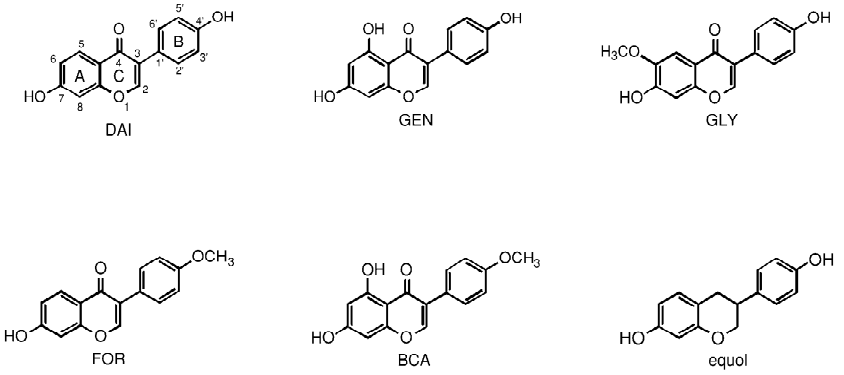

Fig. 1. Chemical structures of common isoflavone phytoestrogens and of the isoflavan equol.

isoflavones and their oxidative metabolites were purified by solid-phase extraction and analyzed as described above for microsomal metabolites. For genotoxicity testing in our laboratory, mammalian cells were cultured and various end-points studied as reported in detail recently [10–12].

3. Microsomal metabolism of various isoflavones

The in vitro metabolism of DAI, GEN, FOR, BCA, GLY and equol was studied with microsomes prepared from rat and human liver. The microsomal metabolites were used for elucidating their chemical structures and as reference compounds for the identification of in vivo metabolites in human urine.

3.1. Daidzein

Analysis by HPLC–MS and GC–MS of the extracts obtained from incubating DAI with hepatic microsomes from aroclor-treated male Wistar rats revealed the formation of four monohydroxylated, four dihydroxylated, and one trihydroxylated DAI metabolite. The chemical structures of all metabolites could be unequivocally identified (Fig. 2), as reported in detail [6]. The major products were 6-HO-DAI, 8-HO-DAI, 5,6-diHO-DAI, 3′,6-diHO-DAI and 3′-HO-DAI and 2-HO-DAI, whereas 3′,8-diHO-DAI, 6,8-diHO-DAI and 3′,5,6-triHO-DAI were present in smaller amounts. Thus, with the exception of 2-HO-DAI, the identified monohydroxylated metabolites represent catechols arising from aromatic hydroxylation of DAI at positions 3′, 6, and 8. Subsequent hydroxylations, again at vicinal positions, lead to the four dihydroxylated DAI metabolites (Fig. 2). Several of the mono- and dihydroxylated metabolites were incubated with catechol-*O*-methyltransferase (COMT)/*S*-adenosyl-L-methionine and gave rise to the respective methylethers, thereby confirming the catechol structure of the DAI metabolites [7]. The aromatic monohydroxylation products 6-HO-DAI, 8-HO-DAI and 3′-HO-DAI were also formed in incubations of DAI with human hepatic microsomes, together with traces of 3′,6-diHO-DAI and 3′,8-diHO-DAI [7].

3.2. Genistein

Incubation of GEN with aroclor-induced rat liver microsomes gave rise to four monohydroxylated and two dihydroxylated metabolites, the structures of

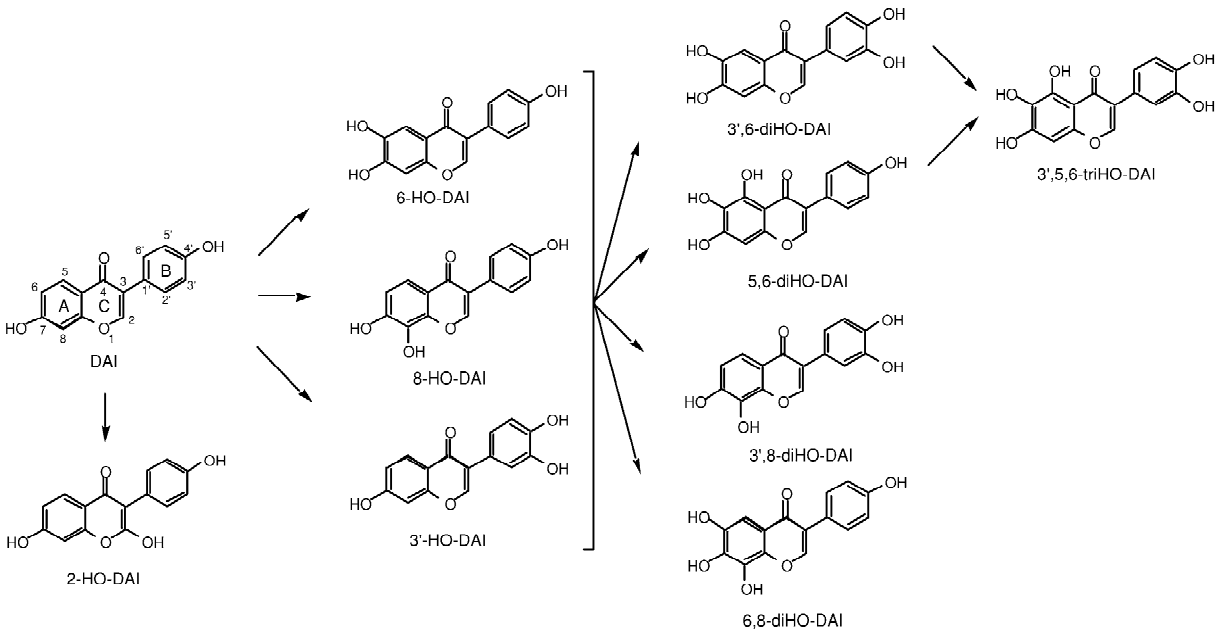

Fig. 2. Oxidative pathways in the metabolism of DAI.

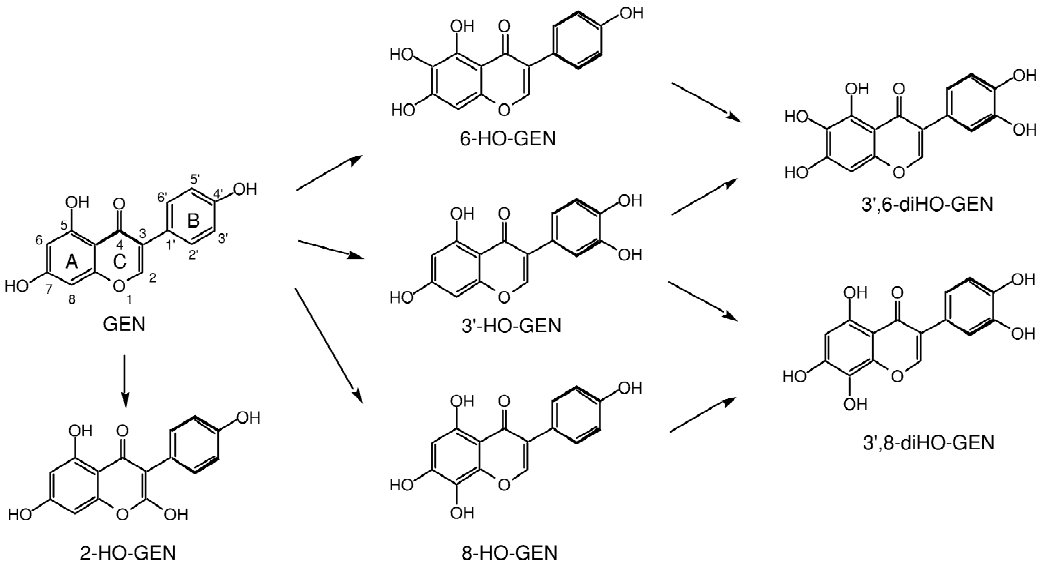

Fig. 3. Oxidative pathways in the metabolism of GEN.

which are depicted in Fig. 3. The predominant metabolites were 6-HO-GEN, 8-HO-GEN, and 3′HO-GEN [6]. As shown above for DAI, all the aromatic hydroxylation products of GEN represent catechols. With human hepatic microsomes, the major metabolites formed were 3′-HO-GEN, 8-HO-GEN, and 6-HO-GEN [7].

3.3. Formononetin, biochanin A, and glycitein

FOR and BCA represent the 4′-methylethers of DAI and GEN, respectively (Fig. 1). In addition to aromatic hydroxylation reactions, the oxidative demethylation at C-4′ is a conceivable metabolic pathway. When FOR was incubated with aroclor-induced rat liver microsomes, it was observed that the major metabolites were DAI, 6-HO-DAI and 8-HO-DAI, whereas 3′-HO-FOR, 6-HO-FOR and 8-HO-FOR were formed in small amounts (Fig. 4). Thus, the oxidative demethylation of FOR to DAI appears to be favored over direct hydroxylation reactions of FOR (Fig. 5). Similarly, the pattern of microsomal metabolites of BCA consisted mostly of GEN, together with some 3′-HO-GEN, 6-HO-GEN and 8-HO-GEN (Fig. 4). The amounts of 3′-HO-BCA, 6-HO-BCA and 8-HO-BCA were relatively small (Fig. 4), indicating again that direct hydroxy-

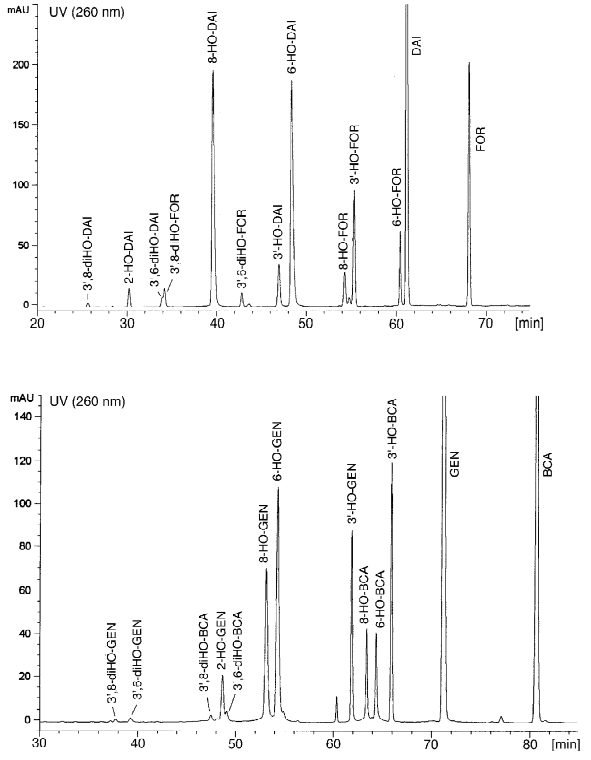

Fig. 4. HPLC profile of the microsomal metabolites of FOR (upper chart) and BCA (lower chart).

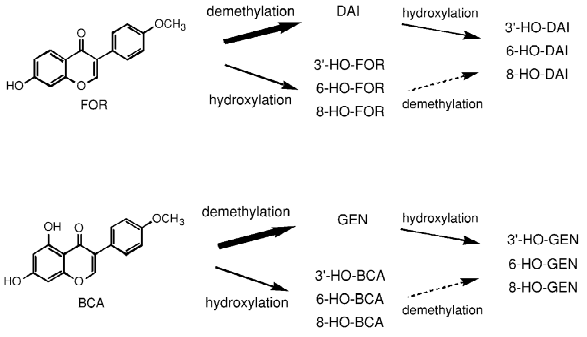

Fig. 5. Oxidative pathways in the metabolism of FOR and BCA.

lation of BCA is less pronounced than demethylation (Fig. 5).

Preliminary studies on the microsomal metabolism of GLY, which is 6-methoxy-DAI, suggest a different metabolic behavior. The two major metabolites formed from GLY were products of aromatic hydroxylation, one of which was identified as 8-HO-GLY, whereas the product of oxidative demethylation of GLY, i.e. 6-HO-DAI, was only present in a small amount (Kulling et al., unpublished data). Thus, direct aromatic hydroxylation appears to be preferred over demethylation in the oxidative metabolism of GLY.

These results are consistent with the recent observation by Setchell et al. [13] that the intake of dietary supplements containing FOR and BCA gives rise to high plasma concentrations of DAI and GEN, whereas ingestion of glycitin (GLY-7-D-glucoside) leads to high plasma levels of GLY.

3.4. Equol

When equol, one of the bacterial metabolites of DAI, was incubated with aroclor-induced rat liver microsomes, seven monohydroxylated and four dihydroxylated products were detected by HPLC–DAD and HPLC–MS analysis (Kulling et al., unpublished data). The predominant metabolite was 3′-HO-equol, whereas 6-HO-equol, 8-HO-equol, 2-HO-equol, 3-HO-equol and 4-HO-equol were formed in smaller amounts. The structure elucidation was mainly based on the mass spectrometric fragmentation of the various metabolites in HPLC–MS and GC–MS, and on reference compounds generated by reaction of equol with tyrosinase/NADH, which catalyzes the ortho-hydroxylation of phenols to catechols. Two HPLC peaks were observed for 4-HO-equol, probably due to the formation of diastereomers. The stereochemistry of the aliphatic hydroxylation products of equol is presently unknown. Hydroxylated metabolites of equol, viz. mostly 3′-HO-equol and 6-HO-equol together with smaller amounts of 8-HO-equol and 4-HO-equol, were also identified in incubations of equol with human hepatic microsomes (Kulling et al., unpublished data).

4. Oxidative isoflavone metabolites in human urine

In order to investigate the formation of oxidative metabolites of the soy isoflavones DAI and GEN in vivo, the urine of three female and three male volunteers was analyzed prior to and after ingestion of soy food. The details of this study have been published [7]. Intake of soy products gave rise to a marked increase in the urinary concentrations of DAI and GEN, which were very low or absent in control urine. In addition to these soy isoflavones and several of their known bacterial metabolites, e.g. dihydro-DAI, dihydro-GEN and equol, several hydroxylated metabolites of DAI and GEN were clearly identified in the urinary extract after conjugate hydrolysis [7]. 3′-HO-DAI, 6-HO-DAI, 8-HO-DAI, 3′-HO-GEN and 8-HO-GEN were already observable in the HPLC profile (Fig. 6), whereas 6-HO-GEN, 3′,6-diHO-DAI, 3′,8-diHO-DAI, 3′,6-diHO-GEN and 3′,8-diHO-GEN were only detected by GC–MS analysis. The identification of all these metabolites was based on cochromatography in HPLC and GC with the respective microsomal metabolites, as well as on comparison of their UV and mass spectra.

In addition to these hydroxylation products of DAI and GEN, which are catechols as discussed above, the urinary extract was searched for the presence of the corresponding methylether metabolites arising from the putative methylation of catechols by COMT. Only the methylethers of 3′-HO-DAI and 3′,6-diHO-DAI could be observed by MS in the

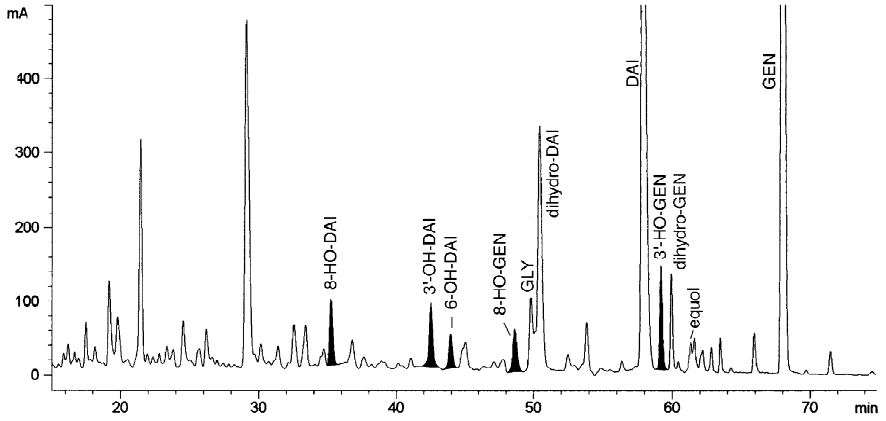

Fig. 6. Characteristic HPLC profile of extracts of human urine after ingestion of soy products. The shaded peaks are hydroxylation products of DAI and GEN.

multiple ion detection mode at concentrations much lower than that of the parent catechols.

Further analysis of the urine by mass spectrometric techniques led to the disclosure of four monohydroxylated equol metabolites, i.e. 3′-HO-equol, 6-HO-equol, 8-HO-equol and 4-HO-equol (cis and trans). Two of these, viz. 3-HO-equol and 4-HO-equol have already been reported previously [14–16]. Preliminary evidence for mono- and dihydroxylated *O*-desmethylangolensin metabolites was also obtained in our studies (Kulling et al., unpublished data).

5. Genotoxic potential of isoflavones

Because dihydroxy compounds structurally related to isoflavones, e.g. the synthetic estrogen DES and the endogenous estrogen E2, exhibit the potential to cause genetic damage [17], our laboratory as well as others have conducted studies on the genotoxic potential of phytoestrogens. Table 1 summarizes data on isoflavones and the coumestan phytoestrogen coumestrol (COM) from our laboratory, using Chinese hamster V79 cells and end-points for genetic damage yielding a positive response to DES or E2. Most of these data are published [10–12]. Striking differences were observed between various phytoestrogens. Whereas DAI was inactive at all end-points, positive effects were observed for GEN, FOR, BCA and COM. All four compounds induced micronuclei in V79 cells; however, further characterization with CREST antikinetochore antibodies showed that the GEN- and COM-induced micronuclei contained

Table 1
Summary of the genotoxic effects of various isoflavone phytoestrogens at different end-points in vitro. The effect of the coumestan phytoestrogen COM and of the endogenous estrogen E2 is included for comparison

End-point	DAI	GEN	FOR	BCA	COM	E2
Micronuclei in V79 cells						
CREST-positive	–	–	++	+++	–	+++
CREST-negative	–	+++	–	–	++	–
Microtubule effects in V79 cells						
Disruption of CMTC	–	–	+	+	–	+
Abnormal mitotic spindle	–	–	++	+++	–	++
Mitotic arrest in V79 cells	–	–	++	+++	–	++
HPRT mutations in V79 cells	–	(+)	–	–	++	nd
Chromosome aberrations in cultured human lymphocytes	–	+++	nd	nd	+++	nd

nd, not determined.

acentric chromosome fragments [10], whereas micronuclei induced by FOR and BCA contained whole chromosomes (Kulling, unpublished data). This indicates a clastogenic potential for GEN and COM, but aneuploidogenic activity for FOR and BCA in V79 cells. The microtubule effects and mitotic arrest observed with FOR and BCA, and the induction of mutations at the HPRT locus [10] and of structural chromosome aberrations by GEN and COM [12] are consistent with this categorization.

Although studies on the genetic toxicity of phytoestrogens are scarce, several other laboratories have also reported genotoxic effects of GEN in cultured cells. For example, Morris et al. [18] observed the induction of micronuclei, HPRT mutations and apoptosis in a human lymphoblastoid cell line treated with GEN, and Pool-Zobel et al. [19] reported on DNA strand breaks induced by GEN in HT29 cells but not DAI. Aberrations of chromosome structures were observed by Abe [20] in human lymphocytes exposed to GEN.

6. Conclusion

Recent studies in our laboratory, summarized in this review, have clearly shown that isoflavone phytoestrogens undergo oxidative metabolism in vitro and in vivo, giving rise to a variety of hydroxylated metabolites, all of which constitute catechols or pyrogallols. It is of considerable interest now to investigate which cytochrome P450 isozymes are involved in the formation of these metabolites and to study their biological properties. As catechol formation is a major pathway in the metabolism of the endogenous estrogen E2 [21], isoflavones may interfere with E2 metabolism if they share the same P450 forms. The introduction of one or two hydroxy groups may increase or decrease the estrogenic and the antioxidative activity of the parent isoflavone and thus modulate two properties associated with the protective effects of these phytoestrogens against certain diseases [22]. Oxidative metabolites may also differ from their parent isoflavones in terms of their genotoxic potential. Studies reviewed above have shown that GEN has clastogenic potential whereas DAI, which carries just one hydroxy group less than GEN, is devoid of this activity. Of particular toxicological interest is the observation that the catechol metabolites of isoflavones appear to be poor substrates of COMT [7]. As the methylation of catechols is commonly considered a detoxification reaction [17,21], a low activity of COMT could lead to high levels of catechols in tissues. If not inactivated efficiently by conjugation, these catechols may undergo redox cycling to form *ortho*-quinones and reactive oxygen species, both of which could damage cellular macromolecules and cause cytotoxicity and genotoxicity. All these aspects of the oxidative metabolism of isoflavones deserve further investigation.

7. Nomenclature

BCA	biochanin A
COM	coumestrol
COMT	catechol-*O*-methyltransferase
DAD	diode array detector
DAI	daidzein
DES	diethylstilbestrol
E2	17β-estradiol
FOR	formononetin
GC	gas chromatography
GEN	genistein
GLY	glycitein
HO	hydroxy
HPLC	high-performance liquid chromatography
HPRT	hypoxanthin phosphoribosyltransferase
MS	mass spectrometry
NADH	reduced nicotinamide adenine dinucleotide
UV	ultraviolet

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