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CHEMISTRY OF BIOLOGICALLY ACTIVE BENZOXAZINOIDS

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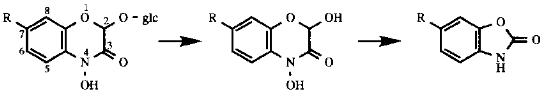
Key Word Index—*Triticum*; Gramineae; grasses; benzoxazinone; electrophile; substituent effect; electrophilic reaction; reaction mechanism.

Abstract—2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and its desmethoxy derivative (DIBOA) are major allelochemicals produced by corn, wheat, rye and related monocotyledons. These benzoxazinone derivatives have a wide variety of biological actions, including antifungal and mutagenic activities. Structure–activity relationships of these compounds and their derivatives (benzoxazinoids), the reactivity of benzoxazinoids with nucleophiles, and the substituent effects of the 7-methoxy and 2-hydroxy groups are discussed in relation to the molecular mechanisms of the biological activities. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

During the course of extensive research aimed at defining the chemical bases of plant resistance to insects, infections, diseases and herbicides, cyclic arylhydroxamic acids (4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-ones, benzoxazinoids) have been implicated as chemical resistance factors [1–4]. Benzoxazinoids are the only group of arylhydroxamic acid derivatives so far detected in plants. Several benzoxazinoids have been isolated from various gramineous species including corn, wheat and rye, and analytical methods for these compounds using HPLC have been developed [5–8].

The predominant benzoxazinoids in corn and wheat are 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**1**, DIMBOA) and its desmethoxy derivative (**2**, DIBOA) (Fig. 1). Though these benzoxazinoids have been detected at high levels (sometimes more than 4 mmole kg^{-1} fresh weight [4, 8]) in several monocotyledons, the compounds exist predominantly as the corresponding stable β-glucosides [2-(2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one)-β-D-glucopyranoside and its desmethoxy derivative] in the living plants (Fig. 1) [3, 9–11]. These glucosides are biologically inactive, but they are enzymatically converted to the active aglycones by β-glucosidase upon plant cell disruption [9, 11–13]. The aglycones (DIMBOA and DIBOA) are further degraded spontaneously to the corresponding benzoxazolinones, 6-methoxy-benzoxazolin-2(3*H*)-one (**3**, MBOA) and its desmethoxy derivative (**4**, BOA) (Fig. 1) [4, 12, 14–18]. Acceleration of this degradation reaction by acylating enzymes was suggested [18]. These benzoxazolinones, as well as some related derivatives including 6,7-dimethoxy-benzoxazolin-2(3*H*)-one and 4-acetylbenzoxazolin-2(3*H*)-one [4, 19], have also been detected in living plants, and have diverse effects, including anti-auxin activity possibly by binding to auxin-binding protein [18, 20, 21], anti-inflammatory activity by inhibiting histamine release [22], and stimulatory activity on reproduction of *Microtus montanus* [23, 24].



R=OMe
R=H

DIMBOA: 1 (R=OMe)
DIBOA: 2 (R=H)

MBOA: 3 (R=OMe)
BOA: 4 (R=H)

Fig. 1. Natural benzoxazinoids.

The roles of DIMBOA and DIBOA in insect resistance [24–26], in herbicide tolerance [4, 24, 27–29], and in allelopathy [24, 30, 31] have been established. Benzoxazinoids also have pharmacological and toxicological properties, i.e. antimicrobial, antifungal, anti-inflammatory, and mutagenic activities [24, 32–37]. A number of synthetic benzoxazinoids have been developed for pharmacological and agrochemical use [38–41].

However, the chemical mechanisms of the actions elicited by these compounds are not well understood. Few chemical studies of cyclic arylhydroxamic acid were performed until the 1970s. In this article, we

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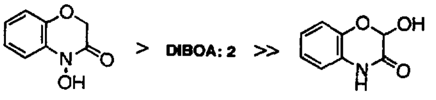
review the benzoxazinoids, with emphasis on their chemical reactivity in relation to their biological actions, and on their structure–activity relationships.

STRUCTURAL REQUIREMENTS OF BENZOXAZINOIDS FOR BIOLOGICAL ACTIVITY AND PROPOSED MOLECULAR MECHANISM

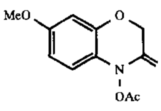
Antifungal and antimicrobial activities of DIMBOA and DIBOA have been well documented [1–4, 24, 32–35]. Because the precursor molecules, i.e. the glucosides, are not biologically active, the 2-hydroxy group of DIMBOA/DIBOA has been considered to be important for biological activity [3, 9, 10, 12, 13]. However, early studies indicated that the 2-deoxy derivative of DIBOA, i.e. 4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**5**, HBOA), shows a higher antimicrobial activity than DIBOA, while the activity of the corresponding 4-deoxy derivative (2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one, **6**) is very weak (Fig. 2) [40]. These results suggest that the 4-hydroxy group of benzoxazinoids is crucial for the biological activity, and imply that the cyclic arylhydroxamic acid group is the functional group responsible for the activity [37, 42]. Therefore, HBOA (**5**) and its 7-methoxy derivative (**7**, HMBOA, Table 1) can be regarded as stable model compounds of the biologically active natural benzoxazinoids.

The arylhydroxamic acid group, like the arylhydroxylamino group, is a typical mutagenic/carcinogenic functional group [43, 44]. As expected, benzoxazinoids show mutagenic activity (Table 1) [36]. From the results in the table, it appears that: (i) both the 7-methoxy group and the 2-hydroxy group enhance the mutagenic activity; (ii) addition of mammalian cytosolic metabolic enzyme system, S-9 Mix, also enhances the activity. It is established that the molecular mechanism of muta-carcinogenesis is based on chemical modification of DNA by an electrophile derived from the mutacarcinogen [43, 44]. Many mutagenic/carcinogenic aromatic amines are known to be successively converted enzymatically to the corresponding hydroxylamines and acyloxyamines, which yield cationic species by heterolytic cleavage of the nitrogen–oxygen (N–O) bond. If this were also the case for the benzoxazinoids, the results shown in Table 1 imply that formation of the cationic species should be facilitated by a 7-methoxy group and/or a 2-hydroxy group, as well as by the addition of S-9 Mix. As S-9 Mix contains acetylation enzymes, one plausible metabolite of strongly mutagenic 4-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**7**, HMBOA) would be 4-acetoxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**8**, AMBOA) (Fig. 3) [37, 42]. In fact, AMBOA reacts with DNA under physiological conditions to give covalently modified DNA with the benzoxazinone ring bound at the C^8-carbon atom of guanine base (**9**, Fig. 4) [42, 45]. The C^8-carbon atom of guanine is one of the nucleophilic centers of DNA. Therefore, the structure of the modified nucleobase indicates that a strong electrophile is derived from AMBOA by heterolytic cleavage of the N–O bond. It is possible that the 7-methoxy group (located at the *para*-position to the hydroxamic acid group), as well as acetylation of the N-hydroxy group, facilitates the N–O bond heterolysis.



HBOA: 5 **6**

Fig. 2. Potency of antimicrobial activity elicited by benzoxazinoids [40].

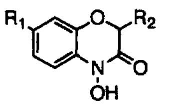


AMBOA: 8

Fig. 3. Possible active metabolite of the mutagenic synthetic benzoxazinoid, HMBOA (**7**).

AMBOA also reacts with amino acid derivatives under physiological conditions (**10–14**, Fig. 5) [42, 46]. The reaction implies the possible occurrence of chemical modification of proteins by biologically active

Table 1. Mutagenicity of benzoxazinoids [36]



			Revertants μmol^{-1}			
			TA100		TA98	
Benzoxazinoid	R_1	R_2	+S-9 Mix	−S-9 Mix	+S-9 Mix	−S-9 Mix
DIBOA (**2**)	H	H	608	285	18	0
DIMBOA (**1**)	CH_3O	OH	223	0	12	0
HBOA (**5**)	H	H	0	0	0	0
HMBOA (**7**)	CH_3O	H	25	94	448	160

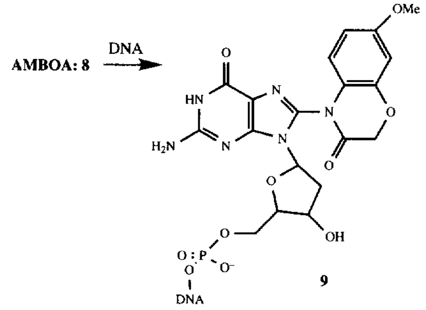


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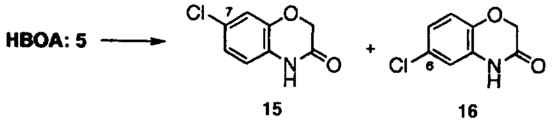
Fig. 4. Biomimetic chemical modification of DNA with AMBOA [42, 45].

Table 2. Chlorination of HBOA



15 16

Reagent	15:16
HCl (aq.)	1:0
CH_3COCl/benzene	0:1
HCl(g)/benzene	0:1
NCS/CH_2Cl_2–CH_3OH	3:1
KCl/CH_3COOH	3:1

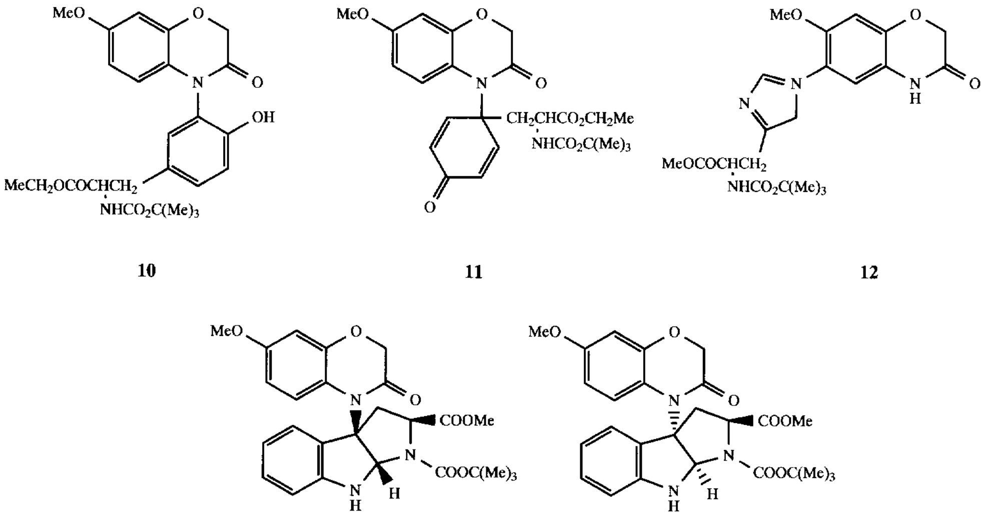
benzoxazinoids *in vivo*. Covalent modification of protein might be the mechanism of antimicrobial/antifungal/insecticidal activity of benzoxazinoids. So, electrophilic nature of the biologically active benzoxazinoids and their reactions with nucleophilic biomolecules could be regarded as key features of the molecular mechanism of their biological activity.

Other chemical properties of benzoxazinoids include catalytic activity for hydrolysis of herbicides such as simazine and diazinone, leading to herbicide tolerance via detoxification of the herbicides, and complex formation with divalent metals, which might be the chemical basis of the toxicity of the benzoxazinoids [47–49].

REACTIVITY OF 4-HYDROXY-2*H*-1,4-BENZOXAZIN-3(4*H*)-ONE (HBOA)

HBOA (**5**) is a synthetic derivative of naturally occurring DIBOA (**2**) with high antimicrobial activity and can be regarded as a superior model compound of DIBOA [40]. Little is known about the chemical reactivity of HBOA, except chlorination and acetylation [50, 51]. Reaction of HBOA with aqueous hydrochloric acid gives 7-chloro-2*H*-1,4-benzoxazin-3(4*H*)-one (**15**) (Table 2). This reaction, i.e. introduction of a nucleophilic chloride ion at the *para* position to the 4-nitrogen atom, can be regarded as a normal reaction similar to the classical rearrangement reaction known as the Bamberger rearrangement [52, 53]. When the source of chloride ion and the solvent are changed to hydrogen chloride and benzene, respectively, 6-chloro-2*H*-1,4-benzoxazin-3(4*H*)-one (**16**) is formed (Table 2). Because position 6 of the benzoxazinone ring corresponds to the *meta*-position to the hydroxamic acid group, this reaction is unusual from the standpoint of the established chemistry of arylhydroxamic acid. Chlorination reactions of HBOA give **15** and/or **16** according to the reaction conditions (Table 2 [37]).

Introduction of a nucleophilic group at the 6-position of the benzoxazinone ring was found by Coutts and Pound in the case of acetylation HBOA with acetic anhydride in acetic acid [51]. In this reaction, HBOA is first acetylated at the oxygen atom to give 4-acetoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (ABOA, **17**), and then the acetoxy group rearranges to the 6-position (**18**) via



10 11 12

13 14

Fig. 5. Structures of reaction adducts of AMBOA with amino acid derivatives [46].

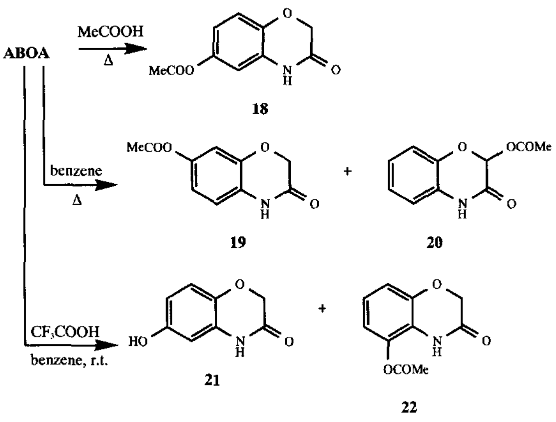


Fig. 6. Rearrangement of ABOA (**17**) [54].

nucleophilic attack with acetic acid, in the high yield of 80%. Investigation of the rearrangement of ABOA under various conditions revealed that positions 2, 5, 6 and 7 can be the electrophilic centre, depending upon the reaction conditions (Fig. 6) [54]. 6-Hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**21**), isolated from the reaction mixture of ABOA with trifluoroacetic acid in benzene (Fig. 6), is interpreted as a hydrolysis product of 6-trifluoroacetoxy-2*H*-1,4-benzoxazin-3(4*H*)-one, which is formed by nucleophilic attack of the trifluoroacetate anion on ABOA. The results shown in Fig. 6 indicate that ABOA is the source of a multi-centred electrophile in which the electrophilic reaction centres can be located at positions 2, 4, 5, 6, 7 and 8 (Fig. 7) [37].

REACTION OF 4-ACETOXY-7-METHOXY-2*H*-1,4-BENZOXAZIN-3(4*H*)-ONE (AMBOA) WITH NUCLEOPHILES

AMBOA (**8**) can be regarded as an active metabolite of HMBOA (**7**) or its model compound, though there is no direct evidence for metabolic formation of AMBOA (*vide supra*). AMBOA is a highly labile electrophile which reacts with various kind of nucleophiles. Its electrophilic reactivity is far higher than that of HMBOA or of ABOA (**17**), because: (i) an acetoxy group is superior to a hydroxy group as a leaving group; (ii) the 7-methoxy group, which is a strongly electron-donating group situated at the *para*-position to the hydroxamic acid group, facilitates heterolytic cleavage of the N–O bond of the benzoxazinone ring. This highly electrophilic nature of AMBOA is reflected in the higher mutagenic activity of HMBOA in the presence of S-9 Mix, as compared with that of HMBOA in the absence of S-9 Mix or HBOA in the presence of S-9 Mix (Table 1).

The electrophilic nature of AMBOA was investigated by analysis of reaction products with various nucleophiles, including phenols, anilines, thiols and heterocycles [37, 42, 55]. These nucleophiles react with AMBOA at various positions according to the nature of the nucleophile.

Phenols always act as carbon nucleophiles with the nucleophilic centre being at an *ortho*- or *para*-position

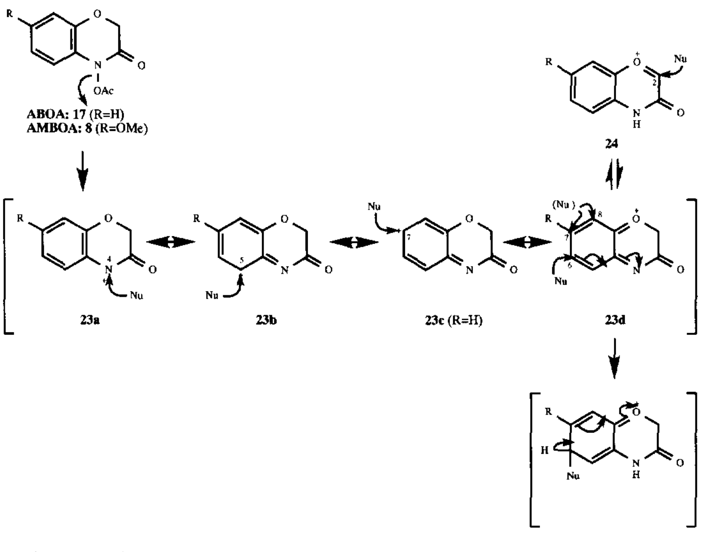
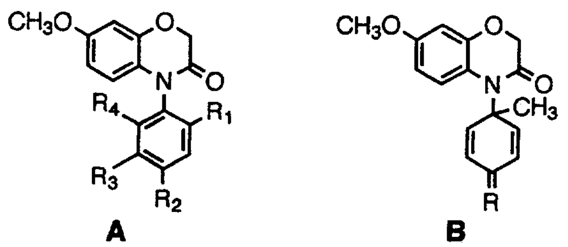


Fig. 7. Multi-centred electrophile formed from ABOA/AMBOA [37, 40].

Table 3. 4-Substituted reaction adducts of AMBOA



		Adduct				
		Type A				Type B
Reactant		R_1	R_2	R_3	R_4	R
Phenol	**25**	H	OH	H	H	—
	26	OH	H	H	H	—
p-Anisole	**27**	OH	H	OCH_3	H	—
m-Cresol	**28**	OH	CH_3	H	H	—
	29	CH_3	OH	H	H	—
	30	OH	H	H	CH_3	—
p-Cresol	**31**	OH	H	CH_3	H	—
	32	—	—	—	—	O
p-Dimethylaminotoluidine	**33**	—	—	—	—	$(CH_3)_2N^+$
Dimethylaniline	**34**	H	$(CH_3)_2N$	H	H	—

of the phenolic hydroxy group, and always react at the 4-position of the benzoxazinone ring (Table 3). Dimethylanilines also act as carbon nucleophiles which react at the benzoxazinone ring's 4-position, but only the *para*-position of the dimethylamino group acts as the nucleophilic centre (Table 3). *Para*-substituted phenols sometimes give *ipso*-attacked adducts such as **11** (Fig. 5) and **32** (Table 3). The latter compound is also formed by reaction of AMBOA with dimethyltoluidine, with the plausible intermediate being **33**.

Though dimethylanilines act only as carbon nucleophiles, unsubstituted aniline acts only as a nitrogen nucleophile. Diazoles and pyridine also act only as nitrogen nucleophiles. They all react with AMBOA at position 6 (Fig. 8). Reaction of AMBOA with a protected histidine (**12**, Fig. 5) can be classified as a reaction of this type.

Other nitrogen heterocycles such as pyrroles and indoles always act as carbon nucleophiles, and react with AMBOA at various positions (Figs 9 and 10). Product analysis of reactions of AMBOA with indoles gives information concerning the nature of the multicentred electrophile derived from AMBOA. It is well established that the most nucleophilic position of an indole skeleton when it acts as a carbon nucleophile is the β-position. Only this position can be the reaction centre which binds to the benzoxazinone ring at positions other than position 4 (**50**–**52**), while both the α- and β-positions of an indole skeleton can bind to the 4-position of the benzoxazinone ring (**45**, **46**, **48** and **49**). Reaction of AMBOA with protected tryptophan (**13**, **14**, Fig. 5) also can be classified as a reation of this type, but in this case, *ipso*-attack followed by cyclization of the formed imine intermediate occurs. The less nucleophilic center of the indole skeleton can react with AMBOA only at its 4-position (**47**–**49**). This indicates that the kinetically most favored electrophilic center of AMBOA is the 4-position. Reactions at other sites, especially at position 6, with nitrogen nucleophiles would be thermodynamically favoured.

Sulphur nucleophiles seem to possess intermediate character between carbon and nitrogen nucleophiles

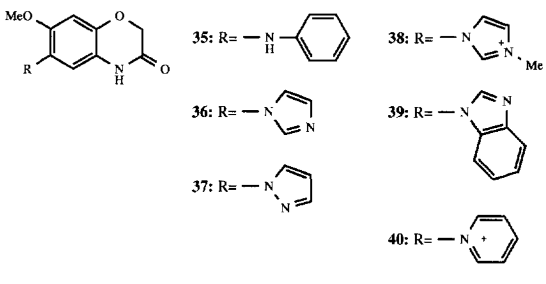


Fig. 8. 6-Substituted reaction adducts of AMBOA with nitrogen nucleophiles.

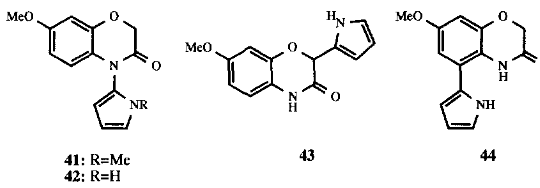
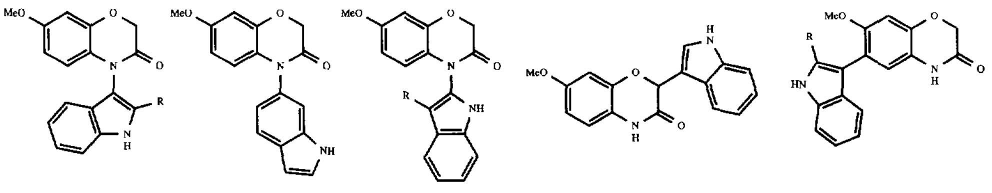


Fig. 9. Reaction adducts of AMBOA with pyrroles.



45: R=H
46: R=Me

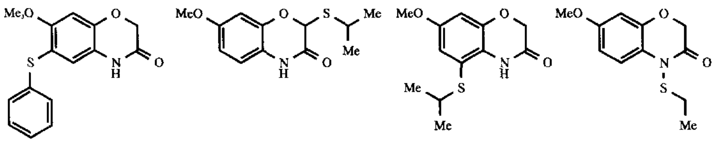
47

48: R=H
49: R=Me

50

51: R=H
52: R=Me

Fig. 10. Reaction adducts of AMBOA with indoles.



53 **54** **55** **56**

Fig. 11. Reaction adducts of AMBOA with sulphur nucleophiles [42].

(Fig. 11). Nucleophilic attack on AMBOA at position 2 can be observed in this case (**54**) and in the case of reaction with pyrrole (**43**). Adducts in which position 2 has been attacked are produced when the cationic species **24** (R=OCH_3, Fig. 7) exists stably, i.e. in reactions with moderate nucleophiles in polar solvents, suggesting that the tautomerism between the two cationic species [**23** (R=OCH_3) and **24** (R=OCH_3)] is the rate-determining step.

Concerning the multi-centred electrophilic nature of AMBOA, the electrophilic reactivity of a benzoquinone monoimine system should be taken into account, because one of the canonical forms of the cation generated by the heterolytic cleavage of the N–O bond of AMBOA [corresponding to the cation **23d** (R=OCH_3), Fig. 7] can be regarded as an *ortho*-benzoquinone monoimine analogue. Electrophilic reactions of *para*-benzoquinone monoimine with some nucleophiles, including phenol and dimethylaniline, have been reported [56]. Various sites of *para*-benzoquinone, including positively charged carbon, nitrogen and oxygen atoms, are attacked by nucleophiles depending on the reaction conditions and the nature of the nucleophiles. *Ortho*-benzoquinone monoimine also reacts with nucleophiles at various sites.

REACTIVITY OF 2,4-DIHYDROXY-2*H*-1,4-BENZOXAZIN-3(4*H*)-ONE: IMPORTANCE OF THE 2-HYDROXY GROUP

We have discussed the chemical nature of biologically active benzoxazinoids, namely HMBOA/AMBOA, regarding them as electrophilic biomolecule-alkylating agents (*vide supra*). It is plausible that naturally occurring benzoxazinoids, namely DIBOA and DIMBOA, also act as biomolecule-alkylating agents. As regards their mutagenic activity (Table 1), enzymatic activation (possibly 4-*O*-acetylation) is necessary for strong activity, as in the case of HMBOA. In this section, we review the chemical reactivity of 4-acetoxy-2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (**57**, AHBOA) on the basis that it can be regarded as an active metabolite or model compound of HMBOA.

As already mentioned, the precursor of HMBOA is the corresponding 2-*O*-*β*-glucoside, which is biologically inactive. Therefore, if the electrophilic nature of AHBOA is the chemical basis of the biological activities, at least in part, the free 2-hydroxy group, but not the 2-*O*-glucosyl group, is expected to facilitate the heterolytic cleavage of the N–O bond. In fact, AHBOA reacts with various nucleophiles, including phenol, under very mild conditions, while the corresponding 2-dehydroxy derivative, ABOA (**17**) does not react with phenol under the same conditions [57]. The substituent effect of the free 2-hydroxy group can be interpreted by the scheme shown as path (**a**) in Fig. 12. Deprotonation of AHBOA followed by ring opening (cleavage of the O^1-C^2 bond) and leaving of the 4-acetoxy group results in an electrophilic *ortho*-benzoquinone monoimine derivative, **58**. These units reactions might occur in a concerted manner. If the ring opening occurs by the cleavage of the C^2-C^3 bond, the reaction would afford an unstable isocyanate, which

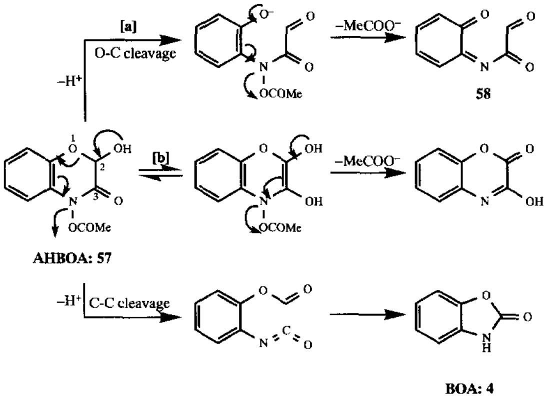


Fig. 12. Possible basis for the substituent effects of the 2-hydroxy group.

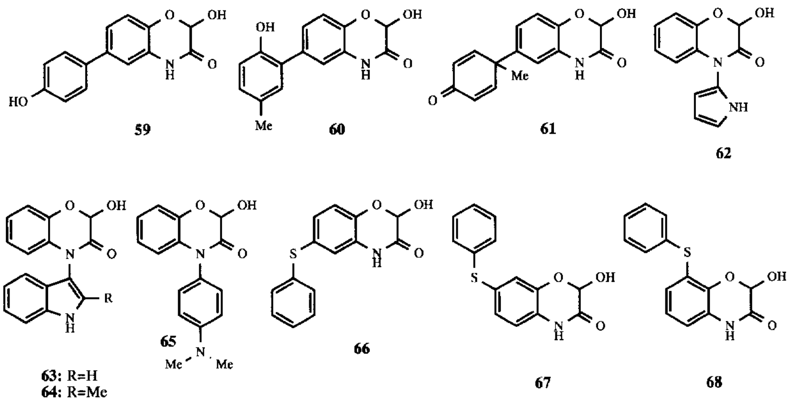


Fig. 13. Reaction adducts of AHBOA with some nucleophiles [57].

is believed to be the intermediate of the decomposition product, BOA (**4**). Other possible interpretations of the substituent effect elicited by the free 2-hydroxy group are (i) a stereo-electronic effect and (ii) enol formation followed by N–O bond cleavage which is concerted with deprotonation (path (**b**), Fig. 12). But the former does not explain the ineffectiveness of the 2-*O*-*β*-glucosyl group, and the latter is implausible, because deuterium-hydrogen exchange experiments indicated no occurrence of keto-enol tautomerization of AHBOA [37, 57].

Though the putative reactive species, **58**, seems to be similar in structure to **23d** (Fig. 7), a difference in the nature of the electrophilic reactions is observed between AHBOA and AMBOA. For example, phenols react with AHBOA only at the 6-position of the benzoxazinone ring, forming a C–C bond (**59**–**61**, Fig. 13) [57]. Reaction with phenols at the 4-position, which corresponds the major reaction site of AMBOA with phenols, is not observed. In the case of reaction with pyrrole, dimethylaniline and indoles, AHBOA reacts at position 4 (**62**–**65**, Fig. 13), as does AMBOA (**34**, **41**, **42**, **45** and **46**, Table 3 and Figs 9 and 10). Thiophenol acts as a sulfur nucleophile in the reaction with AHBOA, giving 6-, 7- and 8-substituted adducts (**66**–**68**, Fig. 13) [57].

REACTION OF A THIO-ANALOG OF ABOA, 4-ACETOXY-2H-1,4-BENZTHIAZIN-3(4*H*)-ONE

Investigation of the reactivity of benzoxazinoids, namely ABOA (**17**), AMBOA (**8**) and AHBOA (**57**), with various nucleophiles revealed that the heterolytic cleavage of the N–O bond of the benzoxazinone ring is facilitated by introduction of a methoxy group at the 7-position (corresponding to the *para*-position to the hydroxamic acid group) or a free hydroxy group at the 2-position (*vide supra*). To develop benzoxazinoids as pharmaceutical/agrochemical agents, other approaches for enhancing the N–O bond heterolytic cleavage would be possible.

One such approach is the replacement of the O^1 atom with a sulphur atom, resulting in 4-acetoxy-2*H*-1,4-benzthiazin-3(4*H*)-one (**69**, Fig. 14) [58]. Compound **69** is expected to possess higher electrophilic reactivity than the corresponding benzoxazinoid, ABOA (**17**), because of the stereoelectronic effect and the high polarizability of the S^1 atom. In fact, **69** reacts with

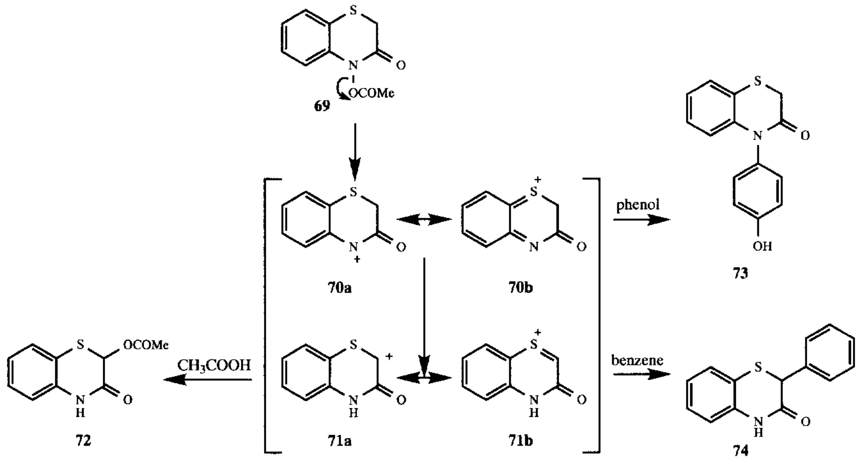


Fig. 14. Reactions of 4-acetoxy-2*H*-1,4-benzthiazin-3(4*H*)-one [58].

phenol under mild very conditions to give the adduct, **73** (Fig. 14), though the corresponding benzoxazinoid analog, ABOA (**17**), does not react with phenol under the same conditions [58]. The electrophilic reactivity of **69** is so high that the compound reacts even with a very weak nucleophile, benzene, to give the 2-substituted analog, **74**. Rearrangement of the 4-acetoxy group of **69** occurs easily to give the 2-substituted analog **72** (Fig. 14).

These reactions can be interpreted as follows: (i) heterolytic cleavage of **69** yields the cation **70**, which is trapped by a strong nucleophile such as phenol to give the 4-substituted analog; (ii) the cation **70** easily tautomerizes to the cation **71** in the absence of a strong nucleophile, and (iii) the cation **71**, whose positive charge is located at the carbon atom adjacent to the sulphur atom, possesses very high electrophilicity and can react even with a weak electrophile to give the 2-substituted analog (Fig. 14) [58]. The nature of the thio-analogue of benzoxazinoids is consistent with established organic sulphur chemistry, and knowledge of the reactivity of the benzthiazinones would be useful for the structural development of biologically active benzoxazinoids/benzthiazinoids.

CONCLUSION

In this article, we have reviewed the chemical reactivity of biologically active benzoxazinoids with emphasis on their reactions with nucleophiles in relation to their biological activity. The biomolecule-alkylating action of benzoxazinoids seems to be the chemical basis for their biological activity, at least in part. The benzoxazinoids afforded a unique multi-centred electrophile, which should be investigated in more detail from the standpoint of pure cationic chemistry.

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