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Haloarene Derivatives of Carbamazepine with Reduced Bioactivation Liabilities: 2-Monohalo and 2,8-Dihalo Derivatives

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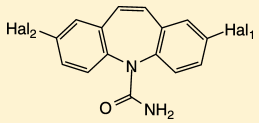
Supporting Information

ABSTRACT: The anticonvulsant carbamazepine **1** is associated with adverse drug reactions (ADRs), including hepatotoxicity; oxidative metabolism of **1** has been implicated in the pathogenesis of the ADRs. We report the synthesis and evaluation of 2-monohalo and 2,8-dihalo analogues of **1** that were intended to minimize reactive metabolite formation via arene oxidation and 10,11-epoxidation. Halo analogues were obtained either by rearrangement of halogenated *N*-arylindoles or from specifically halogenated iminodibenzyl derivatives. In rat hepatocytes, none of the analogues underwent oxidative dehalogenation or glutathione adduction. Some formation of the 10,11-epoxide still occurred, but aromatic hydroxylation was not seen with the exception of 2-fluoro, which allowed minor monohydroxylation. Complete inhibition of aromatic hydroxylation required at least monochlorination or difluorination of **1**. In human liver microsomes, difluoro analogue **5b** underwent 10,11-epoxidation but gave no arene oxidation.



INTRODUCTION

Idiosyncratic adverse drug reactions (ADRs) are a considerable cause of morbidity and mortality in patients,[1] and a significant challenge to the successful development of new drugs and the effective use of existing pharmaceuticals. Although an individual's susceptibility to idiosyncratic reactions is probably multifactorial and can be associated with intrinsic factors such as HLA genotype,[2] the occurrence of ADRs is also linked to a high standard drug dose and reactive metabolite formation.[3,4] Carbamazepine **1** (5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide; Figure 1), which is used widely for the treatment of epilepsy, trigeminal neuralgia, and certain neuropsychiatric conditions,[5] conforms with this pattern.[3] Clinical use has frequently been associated with cutaneous reactions, hematological disorders, and hepatotoxicity.[6] Of patients administered **1**, 30−50% develop ADRs. These adverse reactions have been attributed hypothetically to the formation of one or more reactive metabolites,[7−10] the exact identity of which has yet to be elucidated. Compound **1** undergoes substantial metabolism in patients, with over 30 metabolites having been identified,[11−13] inclusive of sulfur-containing derivatives suggestive of metabolic activation.[11] Definitive evidence of bioactivation, namely, covalent modification of proteins by **1** and production of glutathione (GSH) conjugates of arene oxide intermediates, has been obtained with hepatic microsomal incubations and experimental animals.[9,14,15] Early research indicated a 2,3-substituted arene oxide (Figure 1) might be the reactive intermediate implicated in ADRs,[9,10] but others have also been suggested: *o*-quinones,[14,16] a carbonium ion,[17] a carboxaldehyde,[17] and an iminoquinone metabolite, derived from 2-hydroxycarbamazepine **2**,[18] together with the pharmacologically active carbamazepine 10,11-epoxide **3**, which reacts slowly with GSH in vitro to form two isomeric thioether conjugates.[19,20] The oxidative metabolism of **1** yields primarily **3**, **2**, and 3-hydroxycarbamazepine **4**.[12] Metabolic activation of **1** by human liver microsomes, assessed by the production of numerous GSH conjugates, involves individual or combined pathways of 10,11-epoxidation, arene oxidation, and iminoquinone formation.[20] However, a realistic assessment of a drug's bioactivation requires a complete metabolic system, such as isolated hepatocytes, which effects conjugation reactions that might limit or block entirely certain oxidative pathways. Compound **1**, its phenolic metabolites, and **3** all undergo, variously, *N*- and *O*-glucuronidation.[13]

Structural optimization to preclude the generation of reactive metabolites, as a defensive strategy to attenuate the risk of idiosyncratic ADRs, has become a major consideration in drug development programs.[21] In the present case of **1**, we sought a rational, minimalist, synthetic approach to blocking or limiting metabolic transformations of the dibenzazepine system − arene oxidation and 10,11-epoxidation − known to yield chemically reactive species.[18−20] Derivatives of **1** functionalized at C-10, which often retain pharmacological activity,[22] will be refractory to 10,11-epoxidation but possibly vulnerable to arene oxidation. Comparisons with the biotransformations of the dibenzocycloheptene, cyclobenzaprine, and its 10,11-dihydro analogue (amitriptyline) indicate alkene saturation of **1** will not prevent arene oxidation.[3] Allowing that **3** is a weak electrophile,[19] it was decided one or two monatomic aryl functionalizations intended to block arene oxidation directly, while not necessarily obstructing metabolism to a potentially active 10,11-epoxide, were more likely to be a productive endeavor. This approach might also favor overall retention of pharmacological activity in

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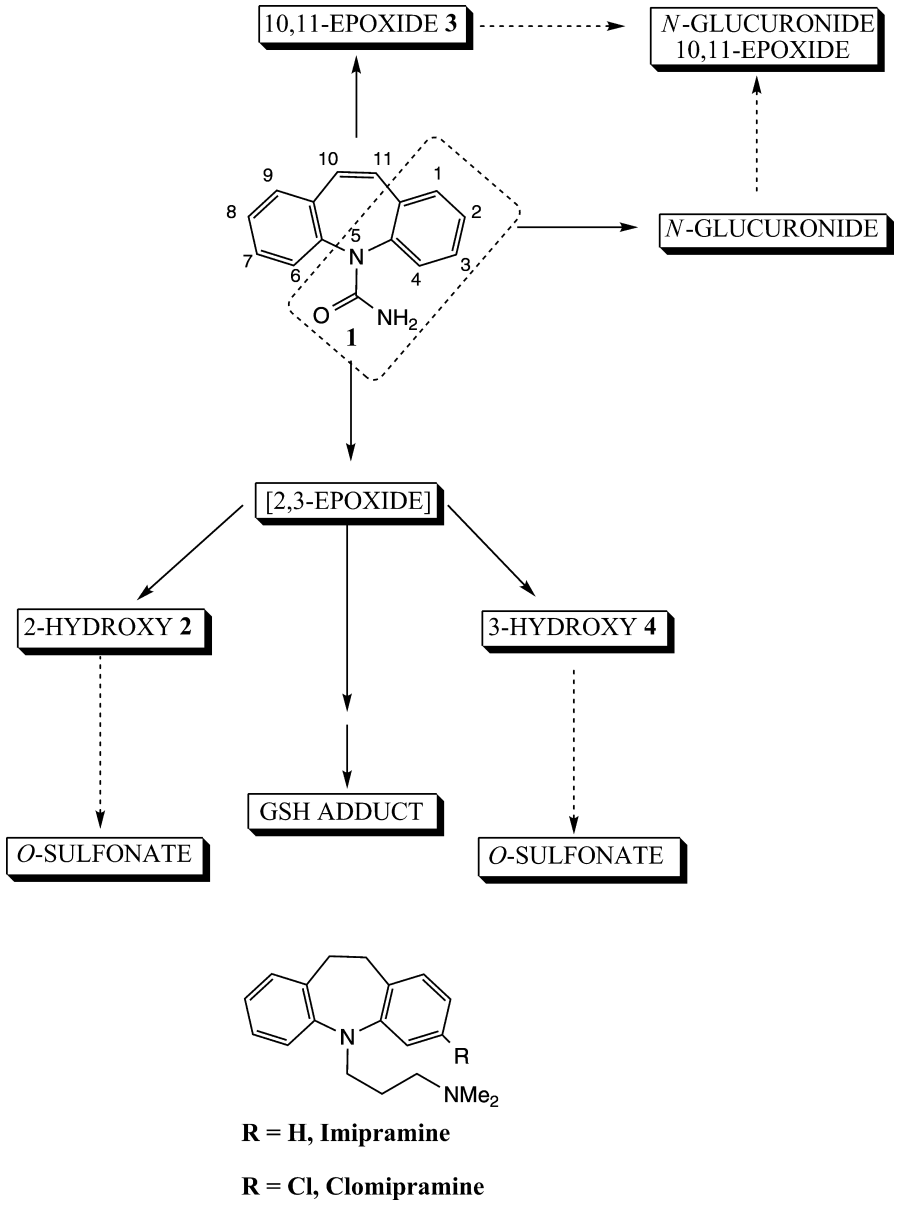

Figure 1. Metabolites of **1** in male rat hepatocytes including 2,3-epoxide intermediate [square brackets]. Stable metabolites were identified by chromatographic comparisons with authentic standards (metabolites **2**–**4**) and/or by LC–MS. Multistep biotransformations are represented by in-line arrows. Hypothetical and alternative metabolic pathways are indicated by broken arrows. The regiochemistry of the GSH adduct is unknown. The regiochemistry of the two *O*-sulfonates is undetermined, but putatively the metabolites are conjugates of **2** and **4**. The related structures of imipramine and clomipramine, referred to in the text, are added for comparison. One aryl ring of **1** and the amide bond (boxed) are the essential pharmacophore elements of the drug for inhibition of neuronal voltage-gated sodium channels in the brain, see Conclusions.

vivo. We additionally bore in mind the possibility these derivatizations will have the independent beneficial effect of nullifying activation of human T-cells by **1** and certain of its metabolites. Both **1** and **3** induce the proliferation of selected T-cell clones from patients hypersensitive to **1**,[23] an effect that has been implicated in the causation of drug hypersensitivity reactions.

Halogenation, and in particular fluorination, of drug molecules is an established strategy for modifying the compounds' metabolic profile to improve medicinal properties.[24] Thus, substitution of a chlorine at C-3 of the 10,11-dihydrodibenzazepine antidepressant imipramine to obtain clomipramine (see Figure 1), which retains pharmacological activity, markedly restrains oxidative bioactivation by human liver microsomes, as evidenced by a reduction in the number of drug GSH conjugates from six to two.[25] However, similar monohalogenations of a dibenzazepine such as **1** do not ensure complete resistance to metabolic oxidation of the aromatic system: the arene oxidation could be redirected to alternative ring positions,[26] or the derivative might be subject to direct oxidative dehalogenation.[21,27] The *N*-carboxamide group might be expected to prevent dehalogenation via an *N*-oxidation that generates a *p*-benzoquinoneimine intermediate, a conjectured mechanism for *p*-fluoroimine structures.[28] Clomipramine appears to be resistant to oxidative dehalogenation in vitro.[25]

We took the preparation of iminostilbene precursors as our starting point for obtaining halogenated and dihalogenated **1**. Although direct aryl halogenation of iminostilbene is not described in the literature, the derivatives may be prepared from ring halogenated acridines and iminodibenzyls by

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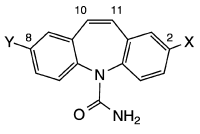
rearrangement and dehydrogenation reactions.[29] Unfortunately, syntheses of substituted acridines often require the use of toxic and moisture-sensitive reagents and are generally low yielding.[30] A safer, more selective route to halogenated **1** is desirable. Only three benzo-halogenated derivatives of **1** have been found in the literature: 3-chlorocarbamazepine, 3,7-dichlorocarbamazepine, and 3,7-dibromocarbamazepine.[31a,b] The iminodibenzyl precursors of compounds such as these have only been synthesized previously by long sequences from chloro-aryl building blocks.[31c] The partially impeded bioactivation of 3-chloroimipramine in vitro[25] suggests the dihalo derivatives might be refractory to aromatic oxidation. However, a C-3 chlorine diminishes anticonvulsant activity of **1** and imipramine in the rat electroshock seizure inhibition test, and 3,7-dichlorination of imipramine abolishes the activity.[31b] Therefore substitutions at C-2 and disubstitutions at C-2 and C-8 were chosen pragmatically, and fluorination was preferred to minimize steric hindrance. They are at least as likely to block metabolic formation of the electrophilic arene oxide precursor(s) of phenols **2** and **4** (Figure 1). Halogens at either C-1 or C-1 and C-9 of **1** were excluded as they are most likely to impede alkene oxidation. Selective synthesis of 2-bromo and 2,8-dibromo iminodibenzyl by bromination of iminodibenzyl with *N*-bromosuccinimide (NBS) is known,[32] but no similar method using *N*-chlorosuccinimide has been disclosed; only chlorination by highly flammable *tert*-butyl hypochlorite has been reported, and it yielded mixtures of regioisomers.[33] Chlorinated iminostilbenes have been synthesized through rearrangement of an appropriate acridine[30] or through polyphosphoric acid cyclization of 1-(4-chlorophenyl)-1*H*-indole.[34] If either method could be effectively extended to selectively form mono- and di-brominated and chlorinated iminostilbenes, it would constitute a marked advance over current methods, which can require seven steps and utilize hazardous and sensitive reagents. We have described the selective synthesis of 2-fluorinated and 2,8-difluorinated iminostilbenes from fluorinated isatins or indoles previously.[35]

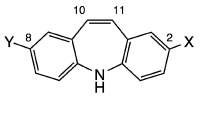
The effects of halogen substitution on the biotransformations of **1** were assessed primarily using freshly isolated rat hepatocytes, a preparation proven to be useful in the investigation of drug metabolism,[36−38] and selectively using human liver microsomes.[9,16,18]

RESULTS AND DISCUSSION

Chemistry. Synthesis of the 2-monohalo and 2,8-dihalo derivatives of **1** (**5a−f**, Chart 1) required first preparation of dibenz[*b*,*f*]azepines **6a−f**, using either of two general methods described below, then attachment of the carboxamide side chain. Although the nucleophilic reactivity of the dibenz[*b*,*f*]-azepines was greatly reduced by halogenation, an effective general method was developed for addition of the urea side-chain.

Chart 1. Structures of Halocarbamazepines Synthesized and Dibenz[*b*,*f*]azepine Precursors





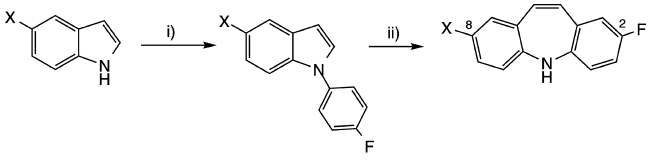
5a X = F, Y = H **5b** X = Y = F **6a** X = F, Y = H **6b** X = Y = F

5c X = Cl, Y = H **5d** X = Y = Cl **6c** X = Cl, Y = H **6d** X = Y = Cl

5e X = Br, Y = H **5f** X = Y = Br **6e** X = Br, Y = H **6f** X = Y = Br

Synthesis of Halo-dibenz[*b*,*f*]azepines. Two methods were employed: either acid-catalyzed rearrangement of an *N*-arylindole or halogenation of iminodibenzyl followed by introduction of the 10,11-double bond. For **6a** and **6b**, we developed the acid-catalyzed rearrangement of *N*-aryl indoles of Tokmakov and Grandberg[34] and prepared various fluoro-dibenz[*b*,*f*]azepines in good yield and purity as described elsewhere,[35] representing the first general synthesis of this class (Scheme 1). Thus 2-fluoro- **6a** and 2,8-difluorodibenz[*b*,*f*]-azepine **6b** were obtained in good yield and in two steps from indole or 5-fluoroindole.

Scheme 1. Syntheses of 2-Fluoro- and 2,8-Difluoro-dibenz[*b*,*f*]azepines[a]

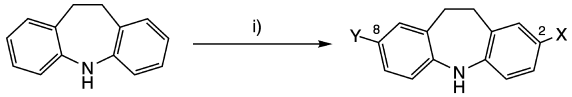


6a, X = H; **6b**, X = F

[a]Conditions: (i) 4-fluoroiodobenzene, CuI, proline, K_2CO_3, $(CH_3)_2SO$, 100 °C; (ii) PPA, 100 °C.[35]

Chloro and bromo analogues **6c−f** were all accessible via direct halogenation of iminodibenzyl **8** (Scheme 2). Using *N*-chlorosuccinimide (NCS) at 20 °C, **7a** and **7b** were obtained with reasonable selectivity from **8** by optimization of equivalents of NCS, solvent, and acid catalyst. Silica gel was an effective catalyst and $CHCl_3$ the optimum solvent; **7a** was obtained in 65% yield with NCS (1 equiv) followed by careful chromatography. *tert*-Butyl hypochlorite has been used for similar chlorinations,[33] but its hazardous nature makes its use unattractive.

Scheme 2. Chloro and Bromo Derivatives of Iminodibenzyl[a]



8

7a X = Cl, Y = H

7b X = Y = Cl

7c X = Br, Y = H

7d X = Y = Br

[a]Conditions: NCS (1−2 equiv) or NBS (1−2 equiv), $CHCl_3$, SiO_2, 0−20 °C; for details, see text.

Using NCS (2 equiv), **7b** was obtained in 80% yield. Other acid catalysts (Amberlyst H^+, trifluoroacetic acid, 1 M HCl) gave no better selectivity, though with 2 equiv of NCS **7b** was always the major product. Chlorination of dibenz[*b*,*f*]azepine produced complex mixtures of products. Analogously, bromination of **8** using NBS essentially as reported by Smith et al.[32] was controllable, giving good yields of **7c** and **7d**. The reactions with NBS were much faster than with NCS, being complete in 10−20 min at 20 °C in $CHCl_3$ using silica catalysis. Dibromo

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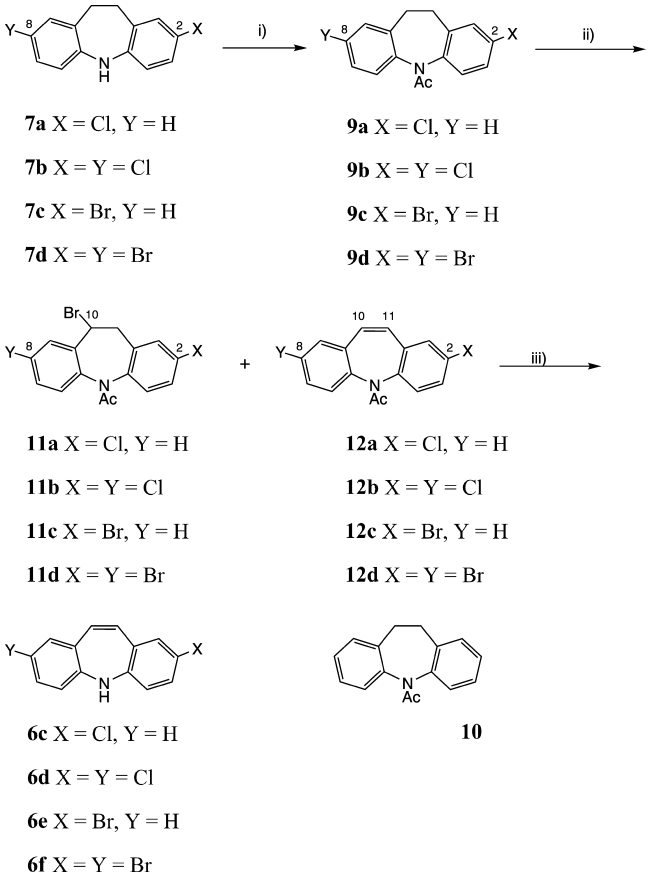
compound **7d** was readily obtained in high yield and purity; **7c** was isolated in 75% yield after chromatography.

To introduce the 10,11-double bond, we used the radical bromination-elimination sequence (Scheme 3) of Schindler,[31a,d] following *N*-acetylation of the amines. The most satisfactory procedure applied to **7a–d** was to use acetic anhydride with DMAP [4,4′-(dimethylamino)pyridine; 1 equiv], giving reproducibly 60–90% yields of *N*-acylated products **9a–d**. Compounds **9a**/**9b** were not easily obtained from *N*-acetyliminodibenzyl **10**,[31d,39] which proved difficult to chlorinate. Using Olah's "superacid" conditions[40] (excess NCS, CF_3SO_3H, heat; milder conditions were ineffective[41]) mixtures of chlorinated products, up to trichloro by MS analysis, were observed.

For the chloro analogues **9a** and **9b**, radical bromination at C(10) was best achieved using NBS (1.5 equiv) with 1,1′-azobis(cyclohexanecarbonitrile) (ACCN) as the initiator in $PhCF_3$. Starting material was fully converted, giving **11a** and **11b** with no overreaction. As well as C(10) bromination, some elimination of HBr occurred in situ giving dibenz[*b*,*f*]azepines **12a** and **12b** directly (Scheme 3). Heating the total product with KOH in EtOH effected both elimination of HBr and deacetylation, affording **6c** and **6d** in high yields;[31d] milder KOH treatment afforded **12a** and **12b**. In practice we progressed the mixtures directly to **6c** and **6d**.

Scheme 3. Chloro and Bromo Derivatives of Dibenz[*b*,*f*]azepine[a]



[a]Conditions: (i) Ac_2O, DMAP, 90–100 °C; (ii) NBS (1.5 equiv), ACCN, $PhCF_3$, heat; (iii) KOH/EtOH, 80 °C.

The Br compounds **9c** and **9d** were similarly progressed, but great care was necessary to avoid side reactions from **9c**. Eventually, using strictly 1 equiv of NBS, intermediates **11c**/**12c** were obtained in good yield with minimum over-bromination, but by LC–MS analysis traces of an isomeric Br compound were also formed. This compound must be due to rearrangement in the radical step, as the precursor was a single isomer. As for the chloro derivatives, we combined elimination with *N*-deprotection, yielding 2-bromodibenz[*b*,*f*]azepine **6e** directly from **11c**/**12c**. The final carbamazepine analogue was purified rigorously by preparative reversed-phase HPLC. By contrast, **9d** was straightforwardly progressed with no side reactions: the **11d**/**12d** mixture with excess KOH/EtOH afforded highly pure 2,8-dibromodibenz[*b*,*f*]azepine **6f**.

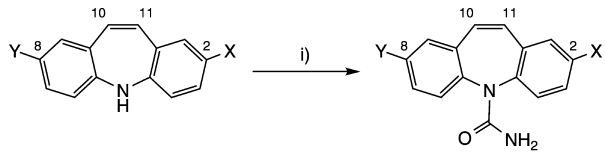
NMR analysis of the *N*-Ac derivatives is complicated by restricted rotation, but the radical bromination step was monitored usefully (see Supporting Information, illustrated for **9d** → **11d** + **12d**). Compounds **11a–d** exhibit a characteristic ABX pattern for the 10/11 protons, with disappearance of the complex CH_2CH_2 signal in **9a–d**. Partial formation of the 10,11-double bond (viz. **12d**) is also apparent.

We later found that the *N*-arylindole route used for the fluoro analogues[34,35] also gave satisfactory yields of **6c** (60%) and **6d** (32%), although slight dechlorination occurred from the dichloro precursor.[42] This method was quite unsatisfactory for the bromo analogues, with only traces (<5%) of **6e** and **6f** isolated from the corresponding precursors.

Carboxamidation. Several methods are known for the carboxamidation of dibenz[*b*,*f*]azepine to yield **1**, especially in patent literature. Phosgene and its preferred modern sources diphosgene and triphosgene[43,44] have been used extensively. We also investigated *N*-chlorosulfonyl[45] and *N*-(trichloroacetyl)isocyanates,[46] reacting the intermediates with NH_3 either in situ or after isolation. Using any of these methods, only halodibenz[*b*,*f*]azepines **6d** and **6f** reacted satisfactorily, owing to limited stability of the intermediates.[42]

The simplest industrial preparation of **1** involves the reaction of dibenz[*b*,*f*]azepine with NaOCN or KOCN,[47] and we applied this to **6a–f** (Scheme 4). The use of acetic acid as the reaction medium is fully satisfactory for **1** (NaOCN, 20 °C, 6 h, 83–89%) but quite unsuccessful for the halo analogues. However, we noted that the reaction of NaOCN with diphenylamine, a weak base with similar pK_a to **6a–f**, was greatly accelerated by using TFA as both solvent and catalyst.[48] These conditions were successful for halogenated analogues **6a–f** (Scheme 4 and Table 1). A steady reaction occurred at 20 °C; even the less reactive fluoro compounds were complete in 12 h. Following addition of toluene and then filtration, the products were isolated in good spectroscopic purity. For the

Scheme 4. Introduction of the Carboxamide Unit via Alkali Metal Isocyanates (Structures as in Chart 1)[a]



[a]Reagents: (i) NaOCN (1.2 equiv), TFA (min. 2 equiv), PhMe, 20 °C.

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Table 1. Conversion of Dibenz[*b*,*f*]azepines to Carbamazepines Using NaOCN/TFA[a]

iminostilbene	temp (°C)	carbamazepine	time (h)	yield (%)
6a	20	**5a**	12	78
6b	20	**5b**	12	76
6c	20	**5c**	6	80
6d	20	**5d**	6	91
6e	20	**5e**	6	89
6f	20	**5f**	6	87

[a]For reaction conditions, see Scheme 4.

metabolic studies, they were purified to 99% purity on a 5−10 mg scale by reversed-phase preparative HPLC.

In conclusion, we developed efficient preparations for all six halo-carbamazepine analogues **5a**−**f**. The dibenz[*b*,*f*]azepine intermediates were obtained by either acid-catalyzed rearrangements of *N*-aryl indoles or direct halogenation of iminodibenzyl followed by introduction of the 10,11-double bond, according to the halogen involved. Direct reaction of the resulting amines with NaOCN in TFA provided an excellent route to the desired halo-carbamazepines in all cases.

Metabolism. The metabolic stabilities of **1** and halogenated derivatives **5a**−**f** were evaluated in freshly isolated male rat hepatocytes incubated with substrate for 6 h. In every case, the maximum number of metabolites detected by mass spectrometry was obtained at the lowest substrate concentration investigated, i.e., 50 μM. Based upon LC−MS analyses (see the Supporting Information) and chromatographic comparisons with authentic standards,[15] **1** was metabolized to the 10,11-epoxide **3** and phenolic derivatives **2** and **4** (Table 2). Formation of **2**, **3**, and **4** was reduced >90% by the non-specific P450 inhibitor 1-aminobenzotriazole[37,38] (data not shown). Additional metabolites of **1** were identified from diagnostic fragmentations in their electrospray mass spectra (see the Supporting Information): the *N*-glucuronides of **1** and **3** (Figure 1), identified by characteristic fissions within the glucuronic acid moiety;[49] two *O*-sulfonates, of unknown regiochemistry but putatively derived from **2** and **4**; and a GSH conjugate of **1** (Supporting Information, Figure S6). The *N*-glucuronide of **3** and corresponding metabolites of **5a**−**f**, were differentiated from alternative monoxygenated structures such as phenolic *O*-glucuronides by the unmistakeable fragmentations of the 10,11-epoxide function (Supporting Information, Tables 1−7). The GSH adduct was of the [M − 2H + GSH] type,[25] formation of which is usually attributed to spontaneous dehydration of the primary dihydrohydroxyl product of GSH addition to an arene oxide.[15] It has been found previously in incubations of **1** with rat hepatocytes.[50] The rat hepatocytes expressed arene oxidation (2- and 3-hydroxylation), 10,11-epoxidation, and *N*-glucuronidation and therefore gave the required coverage of major pathways by which **1** is metabolized in humans. Only one of the major biotransformations of **1** in humans was not represented in the rat hepatocytes, namely, enzymic hydrolysis of **3** to a dihydrodiol.[12,13] This reaction is not catalyzed detectably by rat liver microsomes.[51] Although the 10,11-epoxide might have been a substrate for alternative reactions in rat hepatocytes, specifically, aromatic hydroxylation and GSH conjugation,[19,20] neither dioxygenated derivatives of **1** nor GSH conjugates of the type [M + O + GSH] were found. Metabolites of **5a**−**f** were identified from their mass spectra. All of the derivatives were refractory to oxidative dehalogenation: none yielded a dehalogenated monohydroxyl metabolite.[21,27] They did not produce simple GSH adducts ([M − 2H + GSH]), nor were any of them metabolized to a glutathione adduct of either a dehalogenated ([M − HX + GSH])[52] (representative LC−MS data for **5a** and **5b** are shown in the Supporting Information, Figures S7 and S8), hydroxylated ([M + O − 2H + GSH]),[25] or hydroxylated dehalogenated ([M + O − HX + GSH])[25] species. Only 2-fluoro **5a** was metabolized to a hydroxyl compound, but unlike the hydroxyl metabolites of **1** it was converted entirely to an *O*-sulfonate conjugate, which was found at every substrate concentration (50−1,000 μM). All of the derivatives formed a 10,11-epoxide, an *N*-glucuronide, and an epoxide *N*-glucuronide when incubated at 50 μM. These were the principal metabolites of **1** and **5a**−**f** (Tables 2 and 3). A trend toward suppression of 10,11-epoxidation was evident, this being conspicuous with difluorination (**5b**). No consistent influence on *N*-glucuronidation was discernible. Retention of *N*-glucuronidation at the carboxamide side chain conformed with the observation that C-3 chlorination of imipramine does not block *N*-glucuronidation at the *N*,*N*-dimethylpropan-1-amine side chain.[53] All of the derivatives also yielded an *N*-glucuronide of a 10,11-epoxide, at least at the lowest substrate concentration. The alternative pathways of hydroxylation and *N*-glucuronidation of the 10,11-dihydrodibenzocycloheptene amitriptyline in humans[54] suggest a 10,11-epoxide *N*-glucuronide might be formed from both of its potential precursor metabolites, i.e., via epoxide *N*-glucuronidation and *N*-glucuronide epoxidation. At higher concentrations (≥200 μM) of **1**, **5a**−**d** and **5f**, some of the metabolites were either

Table 2. Metabolic Transformations of 2-Monohalo and 2,8-Dihalo Derivatives (5a−f) of 1[a]

	metabolite[b]					
compd ID[c]	10,11-epoxide	phenols	GSH adduct	*N*-glucuronide	epoxide *N*-glucuronide	*O*-sulfonate(s)
1	√	√[d]	√[e]	√	√	√[f]
5a	√	×	×	√	√	√[g]
5b	√	×	×	√	√	×
5c	√	×	×	√	√	×
5d	√	×	×	√	√	×
5e	√	×	×	√	√	×
5f	√	×	×	√	√	×

[a]Substrates (50 μM) were incubated with freshly isolated male rat hepatocytes for 6 h. [b]The metabolites were identified by chromatographic comparisons with authentic standards (metabolites **2**−**4** of **1**; Figure 1) and additionally or entirely from their LC−MS electrospray ionization mass spectra (see the Supporting Information). [c]See Chart 1. [d]Metabolites **2** and **4**. [e]Regiochemistry of GSH adduct unknown. [f]Two *O*-sulfonates of **1** (regiochemistry undetermined but putatively the metabolites are *O*-sulfonates of **2** and **4**). [g]One *O*-sulfonate of **5a** (regiochemistry unknown).

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Table 3. Metabolites of 2-Monohalo and 2,8-Dihalo Derivatives (5a–f) of 1[a]

	metabolite[b]				
compd ID[c]	10,11-epoxide	*N*-glucuronide	epoxide *N*-glucuronide	*O*-sulfonate I	*O*-sulfonate II
1	0.52 (±0.32)	0.63 (±0.40)	0.24(±0.17)	0.08 (±0.04)	0.32 (±0.25)
5a	0.28 (±0.14)	0.784 (±0.38)	0.15 (±0.09)	NA	0.41 (±0.22)
5b	0.07 (±0.09)	0.18 (±0.06)	0.02 (±0.01)	NA	NA
5c	0.21 (±0.05)	2.04 (±1.06)	0.11 (±0.14)	NA	NA
5d	0.14 (±0.09)	0.22 (±0.16)	0.21(±0.01)	NA	NA
5e	ND	ND	ND	NA	NA
5f	0.19 (±0.14)	0.27 (±0.07)	NQ	NA	NA

[a]Substrates (50 μM) were incubated with freshly isolated male rat hepatocytes for 6 h. [b]Metabolite ratios (metabolite:parent compound; means of three independent determinations ± SD except for **1**, where $n = 13$) were calculated from the UV peak areas (254 nm) of analytes separated by HPLC. The metabolites were identified variously by chromatographic comparisons with authentic standards (metabolites **2**–**4** of **1**) and from their LC–MS electrospray ionization mass spectra (see the Supporting Information). NA = not applicable (metabolite not found). ND = not determined. NQ = not quantifiable.

not detected or were appreciably less abundant. In particular, the GSH adduct of **1** was detected only at 50 μM. The 10,11-epoxide *N*-glucuronide was undetected at 1 mM, 200 μM, and 500 μM in the case of **1**, **5a**, and **5c**, respectively, and 500 μM, 200 μM, and 100 μM in the case of 2,8-difluoro **5b**, 2,8-dichloro **5d**, and 2,8-dibromo **5f**, respectively. Analogue **5b**, as the least heavily substituted derivative that was refractory to arene oxidation in rat hepatocytes, was taken for assessment of the derivatives' bioactivation by human oxygenases, using liver microsomes. The turnover of **1** and **5b**, estimated by LC–UV analysis, was approximately 1%. The selective NADPH-dependent hydroxylation of **1** at C3 by human liver microsomes (Supporting Information, Figure S9) suggested **5b** might undergo the same biotransformation, depending hypothetically on the (unknown) relative extents to which 3-hydroxylation proceeds via 3,4- and 2,3-epoxides. In fact **5b** only underwent 10,11-epoxidation (Supporting Information, Figure S10), the principal biotransformation of **1** in microsomal incubations. As in rat hepatocytes, the 2,8-difluoro substituents effectively deactivated the entire aromatic system against arene oxidation. Neither **1** nor **5b** yielded either a diol, through hydrolysis of its 10,11-epoxide, or dioxygenated metabolites,[16] nor did **5b** undergo oxidative defluorination. Epoxide hydrolysis was not seen because the incubation pH, 7.4, was selected for P450 activity rather than the activity of microsomal epoxide hydrolase, which is maximal at pH 9.5.[51]

CONCLUSIONS

Two series of 2-monohalo and 2,8-dihalo (fluoro, chloro, and bromo) derivatives of **1** were prepared. These compounds were designed to identify the minimum aryl substitution(s), in respect of steric bulk, required for complete inhibition of aromatic hydroxylation of **1**, and therefore, by implication, the formation of electrophilic arene oxide precursors.[9,15,20] This objective was achieved equally with the 2-chloro (**5c**) and 2,8-difluoro (**5b**) derivatives without completely inhibiting 10,11-epoxidation, a biotransformation of **1** that produces pharmacologically active[55] and weakly electrophilic[19,20] **3** (Figure 1). The substituent effects compare favorably with the extensive but incomplete attenuation by a C-3 chlorine of imipramine's bioactivation.[25] The apparently greater influence of a C-2 chlorine on oxidation of the dibenzazepine system might in fact be a corporate result of electronic and steric effects exerted by the halogen and a carboxamide group versus the γ-aminopropyl group in imipramine. Although oxidative dehalogenation is known for numerous and diverse haloaromatics, chlorinated[56] and fluorinated,[21,27,57,58] none of the derivatives of **1** underwent this reaction detectably in rat hepatocytes. The urea *N*-group in these derivatives evidently prevented any dehalogenation initiated by one-electron *N*-oxidation, proposed for certain *p*-fluoroimine structures,[28] which yields a benzoquinoneimine intermediate, precursor to a *p*-hydroxyimine. Exceptionally, the steric hindrance of the 2-chloro and 2-bromo substituents was sufficient to block hydroxylation of both the contralateral benzo ring and the functionalized ring. A similar, though selective, long-range inhibitory influence of aryl-bromine substituents has been seen in a steroid system.[59] Notwithstanding monohalogenation of an aromatic ring can block its metabolic hydroxylation entirely,[60] this familiar design objective of medicinal chemistry is not attained consistently. Arene oxidation occurs, and in some cases extensively,[58] at ring carbons adjacent to and more distant from fluorine- and chlorine-functionalized carbons.[26,57,61–63] Thus the C-3 chlorine of clomipramine (3-chloroimipramine) fails to prevent hydroxylation at C-2 as well as hydroxylation of the contralateral benzo ring.[61,64] Even dihalogenation of a phenyl ring,[56,65–67] or symmetric bilateral difluorination of a dibenzo compound,[57] cannot guarantee complete suppression of arene oxidation. The metabolism of halobenzenes reveals that a bromine, but not a chlorine, at least in monocyclic substrates, impedes hydroxylation at the *ortho* position and partially redirects it to the *meta* position.[62] However, 2-fluoro **5a** was the only derivative to undergo detectable hydroxylation in the hepatocytes. The location of the sulfonated hydroxyl could not be determined, and the possibility of an NIH-shift type fluorine migration,[57,58] mediated by a 2,3-epoxide intermediate, should be considered. Importantly, the inhibition of arene oxidation was essentially selective: not even 2,8-dibromination or 2,8-difluorination of **1** blocked either alkene epoxidation, the major pathway of **1** in humans,[12,13] or *N*-glucuronidation entirely,[13] nor was there any evidence of fundamentally new metabolic pathways enforced by re-directing effects of the halogens.[59] Consequently 2-chlorination and 2,8-difluorination might have negligible unintended impacts on the metabolic fate of **1** in humans. The potential effects of the 2-chloro and 2,8-difluoro substituents on the activity of **1** at an important molecular target of tricyclic anticonvulsant drugs, namely, the neuronal voltage-gated sodium channel, can be analyzed preliminarily by reference to an homology model of the channel's inner pore developed to characterize the inhibitory binding interactions of tricyclic and related anticonvulsants.[68] These substituents might affect directly ligand binding interactions at one of the two

structural components of the drug's pharmacophore (Figure 1). The unsubstituted ring has an aromatic-aromatic interaction with a tyrosine residue. The amino-aromatic hydrogen bonding interactions of the other pharmacophore component, a polar amide in the case of **1**, with a phenylalanine residue can only be disrupted indirectly. The second aromatic ring of **1** fills the pore lumen, hypothetically blocking Na^+ permeation, although it probably has hydrophobic interactions with the protein that might contribute significantly to ligand binding.[68] A voluminous aromatic substituent, as in the case of **5c**, would be expected to impede either the hydrophobic or the aromatic-aromatic interactions, whereas the 2,8-difluoro substituents would not. Notably, however, the 2,3-dichlorophenyl moiety of lamotrigine can be docked inside the model such that the ring interacts with the tyrosine side chain.[68] The restraints of 2,8-difluorination on arene oxidation of **1** were still imposed effectively in incubations with human liver microsomes, confirming the primary objective of selective metabolic resistance had also been achieved in the human context. In patients, **1** is metabolized principally by CYP3A4 and is a pharmacologically significant inducer of this isoform.[69] Histone deacetylase 1 (HDAC1) rather than the pregnane X receptor is required for the induction mechanism in vitro, and **1**, a known inhibitor of HDAC1,[70] retards binding of HDAC1 to the CYP3A4 promoter.[71] Inhibition of the deacetylase activity by **1** may itself have therapeutic value with respect to prostate cancer.[72] Hydrogen bonding, polar, and hydrophobic interactions have been implicated in the active-site binding of HDAC1 inhibitors,[73] certain of which have hydroxamate or benzamide features that resemble the carboxamide moiety of **1**. However, without knowledge of the ligand−protein interactions responsible for preventing binding of HDAC1 to the CYP3A4 promoter, it is not yet possible to deconstruct the possible effects of C-2 and C-8 halo substituents on CYP3A4 induction.

EXPERIMENTAL SECTION

Chemistry: Materials and General Methods. Organic extracts were finally washed with saturated brine and dried over anhydrous Na_2SO_4 prior to rotary evaporation at <30 °C. Moisture-sensitive reactions were carried out in anhydrous organic solvents (purchased from Sigma-Aldrich) under N_2 or Ar atmosphere using oven-dried glassware and basic Schlenk techniques. Reactions were monitored by analytical thin-layer chromatography using Merck Kieselgel 60 F_{254} silica plates and were viewed under UV or by staining with anisaldehyde, vanillin, $KMnO_4$, iodine, or bromocresol green. Preparative flash column chromatography was performed on either VWR Prolabo silica gel or Sigma-Aldrich silica gel (particle size 40−63 Å). Unless specifically stated, separation of compounds was achieved with a product-to-silica ratio of 1:25. Infrared spectra were recorded on a Jasco FTIR ATR spectrometer and are recorded neat or as a ground solid, except where stated. Melting points were recorded using a Bibby-Sterlin Stuart SMP3 melting point apparatus and are uncorrected. High resolution mass spectrometry for the *N*-aryl indoles and fluorinated iminostilbenes was performed by the National Mass Spectrometry Service, Swansea; all other mass spectra (positive- or negative-ion as indicated) were obtained in either electrospray mode (ES) with a Micromass LCT or chemical ionization (CI) mode with a Micromass Trio 1000 using ammonia. Elemental analyses for fluorinated *N*-aryl indoles and iminostilbenes were performed by Mr. Stephen Boyer of London Metropolitan University. Non-fluorinated samples were analyzed by Mr. Steve Apter of the University of Liverpool. ^1H, ^{13}C, and ^{19}F NMR spectra were obtained using a Bruker Avance or a Bruker DPX 400 instrument operating at 400, 101, and 376 MHz, respectively; chemical shifts are reported in ppm (δ) relative to Me_4Si. Coupling constants (J) are reported in hertz.

Preparative HPLC. Aliquots (800 μL) of acetonitrile solutions (10 mg/mL) of the carbamazepine derivative were injected onto the HPLC column without further treatment. The solutions were chromatographed at room temperature on a Knauer Eurospher 100-5 Si column (250 mm × 20 mm i.d.) by gradient elution with acetonitrile (34%, 10 min; 34−66%, 15 min; 66−75%, 40 min; 75−34%, 45 min) in 25 mM ammonium acetate, pH 3.8. The eluent flow rate was 10.0 mL/min. UV detection was set at 298 nm. Instrument management and data processing were accomplished through Clarity software (DataApex, Prague, The Czech Republic).

Analytical HPLC. Aliquots (10 μL) of acetonitrile solutions (0.5 mg/mL) of the carbamazepine derivative were injected onto the HPLC column without further treatment. The solutions were chromatographed at room temperature on a Phenomenex Gemini-NX 5-μm C18 110 Å column (250 mm × 4.6 mm i.d.; Phenomenex, Macclesfield, Cheshire, U.K.) by gradient elution with acetonitrile (15%, 5 min; 15−50%, 20 min; 50−75%, 3 min; 15%, 5 min) in 25 mM ammonium acetate, pH 3.8. The eluent flow rate was 1.0 mL/min. UV detection was set at 298 nm. Instrument management and data processing were accomplished through Chromeleon software (Dionex, Camberley, Surrey, U.K.).

LC−MS Methods. Aliquots (10 μL) of acetonitrile solutions (0.5 mg/mL) of the carbamazepine derivative were injected onto the HPLC column without further treatment. The solutions were chromatographed at room temperature on a Phenomenex Gemini-NX 5-μm C18 110 Å column (250 mm × 4.6 mm i.d.) by gradient elution with acetonitrile/0.1% formic acid (15%, 5 min; 15−50%, 20 min; 50−75%, 3 min; 15%, 5 min) in 25 mM ammonium acetate, pH 3.8. The eluent flow rate was 1.0 mL/min. The chromatographic and mass spectral instrumentation are described under LC−MS for Metabolite Identification. All new compounds were of >98% purity as measured by LC−MS.

General Synthesis of Halo-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepines. 10,11-Dihydro-5*H*-dibenz[*b*,*f*]azepine **8** was dissolved in $CHCl_3$ (ca. 30 mL per mmol of **8**) and pre-dried silica gel (~2.00 g per mmol of *N*-halosuccinimide) was added. The mixture was stirred gently with overhead stirring (or under a Heidolph stirrer fitted with an impellar blade), and the reaction vessel covered with foil to exclude light. *N*-Halosuccinimide (1 equiv for **7a** and **7c**, 2 equiv for **7b** and **7d**) was then added portionwise over 1 h at room temperature. Once addition of reagent was complete, the reaction was left to stir for an appropriate time (see Results and Discussion: Chemistry) before vacuum filtration to remove the silica. After washing with further $CHCl_3$ (2 ×), the combined filtrates were washed with water (2×) and evaporated to dryness. The crude products were purified by column chromatography using EtOAc/hexane gradients to deliver the products **7a**−**d** as pure solids.

2-Chloro-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepine (7a). This compound was obtained in 65% yield as a pale green powder: mp 89−90 °C; ^1H NMR [400 MHz, $(CD_3)_2CO$] δ 7.73 (br s, 1 H, NH), 6.99−7.07 (m, 4 H), 6.90−6.97 (m, 2 H), 6.65−6.75 (m, 1 H, 1-H) and 3.03 (s, 4 H, CH_2CH_2); ^{13}C

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NMR [101 MHz, $(CD_3)_2CO$] δ 144.5, 142.9, 131.4, 131.3, 131.1, 129.6, 129.5, 127.9, 127.8, 120.9, 120.1, 119.3, 36.3 and 35.6; $\nu_{max}(cm^{-1})$ 3379 (w, NH), 1481 (s) and 813 (m); m/z (CI) 230 ([M + H],$^+$ 100%) and 232 (34%); HRMS: found, m/z 230.0734; $C_{14}H_{13}{}^{35}ClN$ (MH^+) requires m/z 230.0707.

Similarly prepared were **7b−d**; see Supporting Information for characterization.

General Synthesis of halo-5*H*-dibenz[*b*,*f*]azepines (6c−f). These were obtained by a three-step sequence from the halo-iminodibenzyls **7a−d**.

(i) The halo-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepines **7a−d** were heated in toluene (5 mL/mmol) with acetyl chloride (1.2 equiv) and DMAP (1 equiv) until reaction was complete. The reaction mixture was cooled and the product extracted by EtOAc/H_2O partition. The organic phase was washed with satd aq $NaHCO_3$ and H_2O and evaporated to dryness. Brief flash chromatography eluting with 15% EtOAc/hexane yielded the *N*-acetyl compounds **9a−d** (60−90%), which were used directly. The NMR spectra of these intermediates were complicated by rotational and fluxional effects, but all showed correct mass data.[42]

(ii) The *N*-acetyl intermediates **9a−d** in $PhCF_3$ (2 mL/mmol) with NBS (1.2 equiv) were heated at 110 °C with ACCN (2 mol %) for 12−24 h with NMR monitoring to ensure complete reaction. At this stage, mixtures of 10-Br compounds **11a−d** and 10,11-dehydro products **12a−d** were present. Products were isolated by cooling, dilution with Et_2O, washing with satd aq $NaHCO_3$ and H_2O, then evaporation.

(iii) The mixtures of products **11a−d** and **12a−d** were dissolved in 1:1 THF/EtOH (20 mL/mmol) and treated with 50% w/v aq KOH (10 mL/mmol), added dropwise over 1 h at 0 °C, then left at 0 °C for a further 1 h before being heated to 80 °C for 12 h. After cooling, H_2O (20 mL/mmol) was added, then 1 M HCl was added to pH 7 followed by extraction with EtOAc (2×). The combined extracts were washed with satd aq Na_2SO_3 and H_2O and then evaporated to give crude halo-5*H*-dibenz[*b*,*f*]-azepines, which were purified by chromatography eluting with EtOAc/ hexane, 1:9, to afford pure **6c−f** as yellow or orange solids. Combined yields for steps (ii) and (iii) are given below; these were excellent apart from **6e**, which was obtained pure by an independent route. The syntheses of the fluoro analogues **6a,6b** have been described previously.[35]

2-Chloro-5*H*-dibenz[*b*,*f*]azepine (6c). This product was obtained as an orange solid in 95% yield on a 4 mmolar scale: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.09−6.94 (m, 1 H), 6.98 (dd, J = 2.3, 8.3 Hz, 1 H), 6.92−6.78 (m, 2 H), 6.83 (d, J = 2.1 Hz, 1H), 6.43 (d, J = 8.4 Hz, 1 H), 6.49 (d, J = 7.8 Hz, 1 H), 6.35 (d, J = 12.0 Hz, 1 H), 6.22 (d, J = 11.7 Hz, 1 H) and 4.91 (br s, NH); ^{13}C NMR (101 MHz, $CDCl_3$) δ 148.0, 146.8, 133.4, 132.1, 131.3, 130.7, 129.9, 129.7, 129.3, 128.9, 127.9, 123.2, 120.3 and 119.3; MS (CI) m/z 228 ([M + H],$^+$ 100) and 230 (39); HRMS: found, m/z 228.0575; $C_{14}H_{11}N^{35}Cl$ (MH^+) requires m/z 228.0575.

Similarly prepared were **6d−f**; see Supporting Information for characterization.

General Synthesis of Halo-carbamazepine Analogues. The appropriate halo-iminostilbene **6a−f** (200 μmol) was dissolved in toluene (2.5 mL), and NaNCO (240 μmol) was added to the solution. At 20 °C, trifluoroacetic acid (240 μmol) was then added; the resulting suspension became clear yellow then colorless, and precipitation of a white solid was observed after 30−60 min. After 6−12 h (see Table 1) the product was filtered off, washed with ice-cold water (2 × 30 mL), and dried, yielding **5a−f** as white solids. All these analogues were purified by the preparative HPLC method described in the general methods section to remove any trace impurities, yielding compounds of >98% purity suitable for metabolic studies.

2-Fluorocarbamazepine (5a). Compound **6a** (0.038 g, 179.90 μmol) was treated according to the general method to yield the product as a white powder after purification (0.035 g, 78%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (dd, J = 3.5, 1.4 Hz, 1 H), 7.22−7.34 (m, 4 H), 7.01 (td, J = 8.3, 2.9 Hz, 1 H), 6.93 (dd, J = 9.1, 2.8 Hz, 1 H), 6.84−6.88 (d, J = 11.6 Hz, 1 H), 6.72−6.77 (d, J = 11.6 Hz, 1 H), 4.95 (br s, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.5 (d, J = 246.9 Hz), 157.4, 139.8, 137.8, 135.9 (br s), 134.7, 130.4 (d, J = 8.1 Hz,), 129.8 (d, J = 4.2 Hz), 129.0, 128.4 (br s), 128.2, 128.0, 125.2, 116.4 (d, J = 22.6 Hz), 115.4 (d, J = 22.2 Hz); HRMS: found, m/z 254.0847; $C_{15}H_{11}N_2OF$ (M^+) requires m/z 254.0855.

2,8-Difluorocarbamazepine (5b). Compound **6b** (0.047 g, 205.04 μmol) was treated according to the general method to yield **5b** as a white solid after purification (0.042 g, 75%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (dd, J = 8.7, 5.2 Hz, 2 H), 7.08−7.14 (m, 2 H), 7.03 (dd, J = 9.0, 2.8 Hz, 2 H), 6.86 (s, 2 H), 4.98 (br s, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.5 (d, J = 247.3 Hz), 157.4, 137.8, 136.5 (d, J = 7.7 Hz), 136.0 (br s), 130.2 (d, J = 8.4 Hz), 129.0, 128.2, 125.2, 116.7 (d, J = 23.0 Hz) and 115.6 (d, J = 22.6 Hz); HRMS: found, m/z 272.0764; $C_{15}H_{10}N_2OF_2$ (M^+) requires m/z 272.0761.

2-Chlorocarbamazepine (5c). Compound **6c** (0.051 g, 223.99 μmol) was treated according to the general method to yield **5c** as a white powder after purification (0.048 g, 79%): ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.43−7.47 (m, 2 H), 7.30−7.42 (m, 5 H), 6.96 (d, J = 11.6 Hz, 1 H), 6.84 (d, J = 11.5 Hz, 1 H), 4.66 (br s, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.8, 142.4, 139.8, 138.5, 136.3, 134.8, 133.3, 131.4, 130.2, 129.8, 129.8, 129.3, 129.1, 128.5 and 128.1; HRMS: found, m/z 270.0563; $C_{15}H_{11}{}^{35}ClN_2O$ (M^+) requires m/z 270.0560.

2,8-Dichlorocarbamazepine (5d). Compound **6d** (0.052 g, 198.37 μmol) was treated according to the general method outlined above to yield **5d** as a white powder after purification (0.055 g, 91%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40−7.42 (d, J = 2.3 Hz, 2 H, H-C), 7.36 (d, J = 1.8 Hz, 2 H), 7.26 (s, 2 H), 6.89 (s, 2 H, H-C10,11), 4.46 ppm (br s, 2 H, $CONH_2$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.3, 138.8, 136.5, 132.7, 132.4, 130.3 and 121.7; m/z (ES+ve mode) 327 (100), 329 (97) and 331(25); HRMS: found, m/z 327.0056; $C_{15}H_{10}N_2{}^{35}Cl_2ONa$ (MNa^+) requires m/z 327.0068.

2-Bromocarbamazepine (5e). Compound **6e** (0.051 g, 187.40 μmol) was treated according to the general method to yield the product as a white powder comprising a 1:1 mixture of isomers (determined by LC−MS; 0.051 g, 89%). The mixture was purified by the preparative HPLC method to remove most of the impurity, followed by separation by the analytical HPLC method outlined in the general methods section to remove the remainder of the closely eluting impurity, yielding **5e** as a white powder (0.024 g): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (dd, J = 8.3, 2.4 Hz, 1 H), 7.50 (d, J = 2.2 Hz, 1 H), 7.44−7.49 (m, 1 H), 7.33−7.40 (m, 1 H), 7.23−7.28 (m, 1 H), 7.12−7.21 (m, 1 H), 6.97 (d, J = 11.4 Hz, 1 H), 6.95 (s, 1 H), 6.85 (d, J = 11.5 Hz, 1 H), 4.46 (br s, 2 H); ^{13}C NMR δ 157.8, 156.6, 139.8,

139.0, 136.7, 134.8, 132.3, 132.1, 130.5, 129.9, 129.8, 128.5, 128.1, 121.3; m/z (CI) 315 ([M + H],$^+$ 94%) and 317 (100%); HRMS: found, m/z 314.0058; $C_{15}H_{11}{}^{79}BrN_2O$ (M^+) requires m/z 314.0055.

2,8-Dibromocarbamazepine (5f). Compound **6f** (0.080 g, 225.05 μmol) was reacted according to the general method to yield **5f** as a white solid after purification by the preparative HPLC method outlined above (0.076 g, 87%): ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.55 (dd, J = 8.4, 2.2 Hz, 2 H), 7.51 (d, J = 2.3 Hz, 2 H), 7.32 (d, J = 8.4 Hz, 2 H), 6.87 (s, 2 H), 4.58 (br s, 2 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.3, 138.8, 136.5, 132.7, 132.4, 130.4, 130.3, 121.7; LC−MS (ES+ve mode), single peak; HRMS: found, m/z 418.9014; $C_{15}H_{10}{}^{81}Br_2N_2ONa$ (MNa^+) requires m/z 418.9017.

Hepatocyte Incubations. Male Wistar rats (150−300 g) were obtained from Charles River Laboratories (Margate, Kent, U.K.). Experiments undertaken were in accordance with the criteria outlined in a license granted under the Animals (Scientific Procedures) Act of 1986 and approved by the Animal Ethics Committee of the University of Liverpool. Hepatocytes were isolated by a modified two-step collagenase perfusion method.[74] The liver was perfused first with wash buffer (10× Ca^{2+}-free Hanks' Balanced Salt Solution, 5.8 mM HEPES, 4.5 mM $NaHCO_3$) for 9 min at 40 mL/min and second with digestion buffer (wash buffer, 0.05% collagenase (w/v), 0.0068% trypsin inhibitor (w/v), 5 mM $CaCl_2$) until the required tissue dissolution was achieved. The liver was removed and rinsed with wash buffer (200 mL) containing DNase I (20 mg). A suspension of the cells combed out of the connective tissue was filtered through nylon mesh (125 μm), resulting in a mixture of parenchymal and non-parenchymal cells. The cells were purified by sequential washing and centrifugation (50g, 2 min), once with wash buffer containing DNase I and twice with wash buffer itself. They were finally resuspended in incubation buffer (wash buffer, 1 mM $MgSO_4{\cdot}7H_2O$). Viability was assessed through trypan blue exclusion (20 μL:100 μL cells) and only cells of a viability ≥80% were used. Suspensions of hepatocytes (2×10^6 cells/mL, 6 mL total) were incubated at 37 °C in an orbital incubator with **1** and the haloarene analogues (typically, 50−1000 μM; added in methanol, 1% final volume) for 6 h. The 2-bromo **5e** was incubated only at 50 and 200 μM (added in acetonitrile, 1% final volume) because of a limited stock of this analogue. Incubations were terminated with acetonitrile (6 mL) and stored at −20 °C until analyzed. Supernatants obtained by centrifugation (2200 rpm, 10 min) were concentrated under a stream of N_2 at room temperature. The aqueous residue was loaded onto a Sep-Pak C18 solid-phase extraction cartridge (Waters Ltd., Hertfordshire, U.K.). The cartridge was then washed with water (3 mL) and methanol (3 mL). The methanol fraction was evaporated to dryness under N_2 and reconstituted in methanol/water (1:1, v/v; 500 μL). Aliquots were injected onto HPLC/UV (50 μL) and LC−MS (5−50 μL; 30−50 μL for the 50-μM incubations with hepatocytes) systems. The 50-μM incubations were the only ones that contained all of the drug metabolites that were identified and were taken for metabolite identification by LC−MS. This concentration falls within the therapeutic range of **1** (17−51 μM).[75,76]

Microsomal Incubations. Human liver microsomes pooled from 150 donors with equal numbers of males and females were obtained from BD Biosciences (Woburn, MA). Substrate (**1** or **5b**, 50 μM) and NADPH (1 mM) were incubated with the microsomes (protein concentration, 1 mg/mL) in potassium phosphate buffer, pH 7.4, (0.1 M, 1 mL) for 60 min at 37 °C. NADPH was omitted from the control incubations. Each incubation was terminated by addition of an equal volume of ice-cold acetonitrile, left overnight at −20 °C, and centrifuged at 2200 rpm for 10 min at 4 °C to sediment the protein precipitate. The supernatant was evaporated to dryness at 37 °C under nitrogen and reconstituted in methanol/water (1:1, v/v; 200 μL). Aliquots (50 μL) were injected onto the LC−MS and HPLC−UV systems.

LC−MS for Metabolite Identification. The LC−MS system consisted of a PerkinElmer series 200 pump and autosampler (Norwalk, CT) connected to either an API 2000 (analysis of hepatocyte incubations) or 4000 Qtrap (analysis of microsomal incubations) mass spectrometer (AB Sciex, Foster City, CA) equipped with an electrospray ionization source. Separation of parent compounds and their metabolites was achieved at room temperature on a Phenomenex Gemini-NX 110 Å C18 column (5 μm, 4.6 mm × 250 mm) using solvent A (10 mM ammonium acetate, pH 3.8) and solvent B (acetonitrile). At a flow rate of 1.0 mL/min, the initial eluent composition (15% solvent B) was held constant for 5 min followed by an increase to 50% solvent B over 20 min and a further increase to 75% over 15 min. The eluent composition was returned to its initial proportions over 3 min and finally held for a 2-min re-equilibration period. The column eluate was split, and approximately 15% was routed to the LC−MS interface. The mass spectrometer was set for full scanning (m/z 100−1000; 5 s) and was operated in positive-ion mode. API 2000 operating parameters were as follows: source temperature, 400 °C; electrospray capillary voltage, 5.0 kV; desolvation potential, 60 V; source gas, 15 (arbitrary unit); and heater gas, 75 (arbitrary unit). The 4000 Qtrap operating parameters were as follows: source temperature, 450 °C; electrospray capillary voltage, 5.5 kV; desolvation potential, 100 V; source gas, 50; and heater gas, 50. The mass spectrometry data were acquired and analyzed using Analyst software (AB Sciex). Metabolites of **1** and its halogenated derivatives **5a−f** were identified preliminarily by comparing total and selected extracted ion chromatograms of control (substrate or co-factor free) and test incubations. They were subsequently characterized from their diagnostic in-source fragmentation.

HPLC−UV for Metabolite Quantitation. The LC−UV apparatus consisted of a Dionex Summit HPLC System (Dionex P580 pump and ASI-100 autosampler; Dionex, Camberley, Surrey, U.K.) connected to a Dionex UVD170S UV detector (254 nm). Parent compounds and metabolites were resolved at room temperature on a Gemini-NX 5-μm 110 Å C18 column as described above. They were identified by reference to authentic standards (parent compounds and **3**) and the LC−MS analyses. Absorbance data (analyte peak areas) were processed by Chromeleon software version 6.8 (Dionex).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Characterization data for compounds **7b−d** and **6d−f**. NMR spectrum showing radical bromination-elimination transformation of **9d**. Mass chromatograms of monoxygenated metabolites of **1** and halogenated derivatives **5a−c** in rat hepatocytes, the GSH adduct of **1** in rat hepatocytes, and monoxygenated metabolites of **1** and **5b** in human liver microsomes. Electrospray mass spectra of the metabolites of **1** and **5a−f**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ACCN, 1,1′-azobis(cyclohexanecarbonitrile); ADRs, adverse drug reactions; GSH, glutathione; NBS, *N*-bromosuccinimide; NCS, *N*-chlorosuccinimide; DMAP, (4,4′-(dimethylamino)-pyridine

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