Antitumour benzothiazoles. Part 4.[1] An NMR study of the sites of protonation of 2-(4-aminophenyl)benzothiazoles

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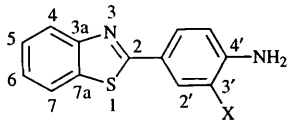
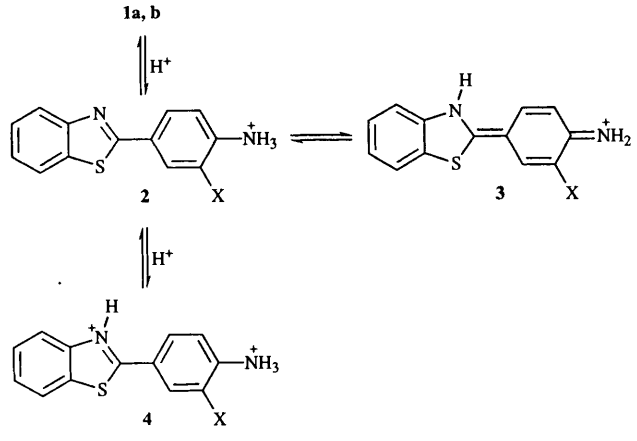
The behaviour of the series of antitumour 2-(4-aminophenyl)benzothiazoles 1a–e in acid solution has been probed by ^{13}C and ^{15}N NMR methods and the sites of protonation determined. The parent compound 2-(4-aminophenyl)benzothiazole 1a and the 2-(4-amino-3-methylphenyl) analogue 1b undergo initial protonation at the exocyclic amino group followed by protonation at the N-3 atom of the benzothiazole nucleus: those analogues with a 3′-halogen substituent, compounds 1c–e, protonate initially at N-3. These observations have potential pharmaceutical significance.

Introduction

Although having a relatively simple structure, the benzothiazole **1a** exhibits subtle and selective activity against human breast tumour cell lines *in vitro*.[1–3] Introduction of a methyl **1b** or



1a X = H
1b CH_3
1c Cl
1d Br
1e I



halogen substituent **1c–e** into the 3′-position confers exceptional potency against a broad spectrum of breast, ovarian, lung and renal cell lines: for the tolyl compound **1b** this activity extends to *in vivo* inhibition of a panel of human oestrogen receptor-positive and -negative breast tumour xenografts growing in nude mice.[1]

The effects of solvent and pH on the absorption and fluorescence spectra of 2-(4-aminophenyl)benzothiazole **1a** and its 2-(2-aminophenyl)- and 2-(3-aminophenyl)-isomers,[4] the *N*,*N*-dimethyl analogue [5] and related 2-(4-aminophenyl)pyridothiazoles [6] have been studied previously. Notably, the long wavelength absorption of the amine base **1a** at 333 nm in cyclohexane is red-shifted to 392 nm in the monocation. The presence of isosbestic points in the spectra indicate that only two species, base and monocation, are involved in the equilibrium (corresponding to a pK_a of 3.0)[4] and the bathochromic shift induced on protonation implies the development of a more extended chromophore than that represented by the ammonium cation **2a**. This observation has been rationalised by invoking a substantial contribution in the ground state by the iminium species **3a** protonated at N-3.[4]

During our evaluation of the physical and pharmaceutical properties of the compounds **1a** and related dimeric structures,[7] we observed that solutions in dimethyl sulfoxide (DMSO) developed intense bright yellow colours on addition of trifluoroacetic acid (TFA) or DCl. Qualitatively similar, but less intense, colour changes were noted for the three halide derivatives **1c–e**. We also found that it was possible to selectively prepare mono or dihydrochloride salts of **1a**,**b**,**d** by exposing the free bases either to aqueous HCl or dry HCl gas. Since the propensity of weakly basic drugs to form salts is of prime importance in predicting absorption, bioavailability and metabolism, and may critically influence formulation design and selection of a suitable route of drug administration, the sites of protonation of these bases have been investigated by ^{13}C and ^{15}N NMR spectroscopy.

Results and discussion

Assignment of all ^1H and ^{13}C resonances

Assignment of all the ^1H and ^{13}C resonances was achieved by standard 1D and 2D NMR experiments: DEPT, SIMPLE,[8] COSY, HETCOR and COLOC [9] optimised for ^3J connectivity. The proton assignments have been published elsewhere [1] and the full ^{13}C assignments are presented in Table 1. The symmetry of the benzothiazole nucleus means that unequivocal assignment of all the ^1H and ^{13}C resonances is dependent on the assignment of C-3a and C-7a. These carbons show equivalent patterns of cross peaks in the COLOC spectrum but the chemical shifts are 20 ppm apart and show remarkably close agreement with values for benzothiazoles quoted in the literature.[10,11] These assignments are in accord with substituent electronegativity with ^{13}C adjacent to nitrogen (C-3a) downfield of ^{13}C adjacent to sulfur (C-7a). Furthermore, in the 6-methyl analogue a small upfield shift relative to **1a** was observed for C-3a whereas C-7a was unaltered—an observation consistent with the assignment.

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Table 1 δ_C Assignments for 2-(4-aminophenyl)benzothiazoles **1a–e**[a]

Compound	C-2	C-3a	C-4	C-5	C-6	C-7	C-7a	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
1a	169.0	154.8	122.6	127.1	125.2	122.7	134.6	121.0	129.7	114.5	153.0	114.5	129.7
1b	168.2	153.9	121.7	126.1	124.2	121.8	133.7	120.3	129.1	121.0	150.3	113.6	126.5
1c	166.5	153.6	122.0	126.3	124.6	121.9	133.8	121.2	127.7	117.0	147.7	115.1	127.3
1d	166.3	155.6	122.0	126.3	124.6	121.9	133.8	121.7	130.9	107.0	148.8	115.0	127.8
1e	166.1	153.6	122.0	126.3	124.6	133.8	133.8	122.3	137.3	82.4	151.5	113.8	128.5

[a] Benzothiazoles 0.3 M solution in $(CD_3)_2SO$ at 296–298 K.

Sites of protonation

For benzothiazoles **1a,b** the first cation is formed at the exocyclic amino group. Thus addition of DCl to the $(CD_3)_2SO$ solution induced a large upfield shift in the C-4′ resonance and a smaller downfield shift in the C-3′/5′ resonance whilst all other signals remained relatively unmoved. Furthermore, when DCl was added to a $(CD_3)_2SO$ solution of **1a** so that the integral of the NH_2 resonance was reduced from 2 H to about 1 H, splitting as seen in the SIMPLE experiment was only observed for C-4′ and C3′/5′, the signals from C-2 and C-3a remained sharp. This shows clearly that the first protonated species is **2a** where C-4′ bears the ammonium group. This result was confirmed by ^{15}N NMR spectroscopy, see below.

In neat TFA, conditions under which it was supposed protonation would be complete, both ^1H and ^{13}C spectra were difficult to interpret. The ^{13}C spectra were sufficiently different from those in DMSO that assignment by analogy was only possible for a few resonances. Full assignment by 2D methods was impossible because of coalescence of signals in the proton dimension; the SIMPLE experiment did not work in TFA because of the fast proton exchange rate. From the considerable shifting of the resonances it was likely that a second protonation had occurred to give the dication **4a**.

To overcome the assignment problem and more closely define the conditions under which a second protonation might occur, a full titration, monitored by ^{13}C NMR spectroscopy, was undertaken. The poor solubility of the drugs in aqueous solution precluded the use of aqueous buffers and a system of $(CD_3)_2SO$ and TFA was adopted, which allowed sufficiently concentrated drug solutions so that the NMR experiments took between 5 and 30 min.

Titrations

For benzothiazoles **1a,b**, as the first 10 equiv. of TFA were added, large chemical shift changes were observed for the carbon atoms *ipso*, *ortho* and *para* to the 4′-amino group. When between 10 and 30 equiv. of TFA were added, there was a plateau with only a shallow drift in chemical shift similar to the solvent effects observed for the $(CD_3)_2SO$ resonance. At 30 equiv. shifting indicative of a second protonation began with large chemical shift changes for carbon atoms of the benzothiazole ring, most notably C-2 and C-3a. (See Fig. 1 and Table 2.) The second protonation was essentially complete after addition of 80 equiv. of TFA, the very shallow titration curve being indicative of the weakness of the base involved.

The pattern of chemical shift changes during the first protonation of **1a**, particularly the large upfield shift of C-4′, is typical of protonated anilines.[12] For benzothiazole **1a**, C-4′ showed a shift change of −15.5 ppm; C-2′/6′ and C-1′ showed shift changes of +8.9 and +8.4 ppm, respectively. There were remote chemical shift changes in the benzothiazole ring consistent with electron withdrawal from the ring transmitted through N-3 of the benzothiazole:[10] these were small but the shapes of the titration curves were distinctly different from those due to solvent effects.

The fact that it was C-2 and C-3a that shifted the most markedly in strong acid showed that the second protonation

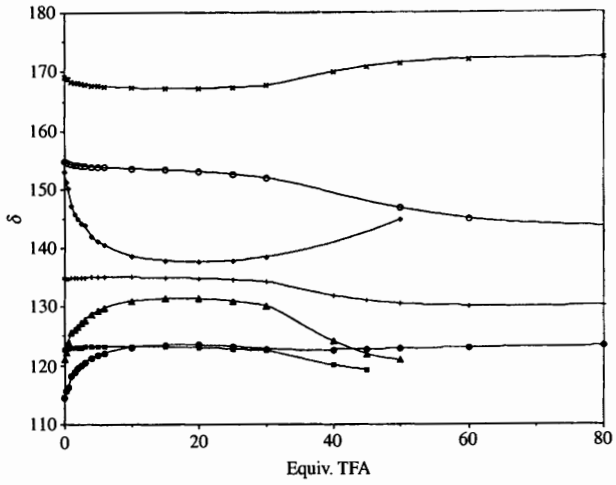
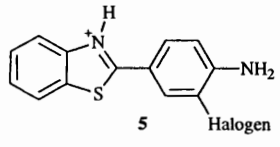


Fig. 1 Plot of ^{13}C chemical shift against concentration of TFA for **1a**; C-2 (×), C-3a (○), C-7 (■), C-7a (+), C-1′ (▲), C-3′/5′ (●), C-4′ (◆). C-4, C-5, C-6, C-2′/6′ gave almost flat plots and have been omitted for clarity.

of **1a,b** occurred at N-3 (**4a,b**). The significant chemical shift changes during the second protonation of **1a** were C-2, + 5.1 ppm; C-3a, −11.4 ppm and C-4′, *ca.* +10 ppm, with smaller changes for C-7, C-6 and C-5. The N-3 lone pair is orthogonal to the π-system of the benzothiazole ring so the electronic effects of protonation are generally inductive. In relation to the benzo ring of the benzothiazole, N-3 behaves as an exocyclic substituent and the *ipso* shielding effect is apparent on C-3a. The downfield shift of C-2 suggests that the C-2 to N-3 bond is highly polarised with partial positive charge on C-2, stabilised by the sulfur atom. The strong resonance connection between C-4′ and N-3 is demonstrated by the large downfield movement of C-4′ during the protonation of N-3 indicating the shifting of electron density back towards the thiazole ring.

For amines **1c–e**, a distinctly different protonation pattern was observed. At low TFA concentrations little perturbation was observed, for example, the C-4′ resonances in Fig. 2. Between 25 and 60 equiv. of TFA, chemical shift changes consistent with protonation of N-3 were seen. Up to 100 equiv. of TFA there was no evidence for even partial protonation of C-4′ (100 equiv. of TFA corresponded to 9.08 M, neat TFA is 12.96 M). Despite this, it was possible to prepare a dihydrochloride salt of **1d** by passing dry HCl gas through an ethyl acetate solution of the free base.

The behaviour of C-2, C-3a and C4′ for **1c–e** closely mirrored that seen during the second protonation of **1a,b** with C-2 and C-3a showing chemical shift changes of about +5 and −11,



5 Halogen

Table 2 ^{13}C Chemical shift changes on *N*-protonation for a series of 2-(4-aminophenyl)benzothiazoles **1a–e**

Compound	Site of protonation	$[TFA]_{50}$	$\Delta\delta$ on protonation[a]											
			C-2	C-3a	C-4	C-5	C-6	C-7	C-7a	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
1a	NH_2	0.4								+8.4		+8.9	−15.5	+8.9	
	NH_2 and N-3	6.2	+5.1	−11.4									*ca.* +10		
1b	NH_2	0.7								+10.8		+10.7	−13.3	+9.6	
	NH_2 and N-3	6.3	+6.6	−9.4				+	−5.5	−11.0		−	+14.0	−	+
1c	N-3	5.8	+6.4	−11.6	−4.1		+		−4.4	−6.2			+3.9		
1d	N-3	5.7	+	−11.4	−4.0	+	+	−6.0	−4.4				+4.0		
1e	N-3	4.9	+	−11.4	*b*	+	+	*b*	−4.5				+4.0		

[a] $\Delta\delta$ for $(CD_3)_2SO$ across the TFA concentration range was −4.0 ppm; thus $|\Delta\delta| < 4.0$ have been ignored. For some carbons, plots of δ *vs.* [TFA] gave typically sigmoidal titration curves from which the chemical shift change on protonation and concentration of TFA to give 50% protonation, $[TFA]_{50}$, were determined. The values of $[TFA]_{50}$ quoted are the mean for the compound converted to mol dm^{-3}. Other carbons showed a drift in chemical shift and where $|\Delta\delta| \geqslant 4.0$ these are marked + and − as appropriate. [b] Peaks too broad or not assigned.

Table 3 ^{15}N NMR data for 2-(4-aminophenyl)benzothiazole **1a**

Solvent[a]	N-3[b]	NH_2[b]
A	−87.5 (+ve, s)	−311.0 (−ve, t)
B	−85.5 (+ve)	−325.0 (−ve)
C	−193.9 (*c*)	−329.8 (*c*)

[a] Solvents: A, $(CD_3)_2SO$; B, $(CD_3)_2SO$ with 10 equiv. TFA; C, TFA. [b] Effect of full ^1H NOE in brackets. [c] Significantly reduced signal to noise, both peaks phase in the same direction.

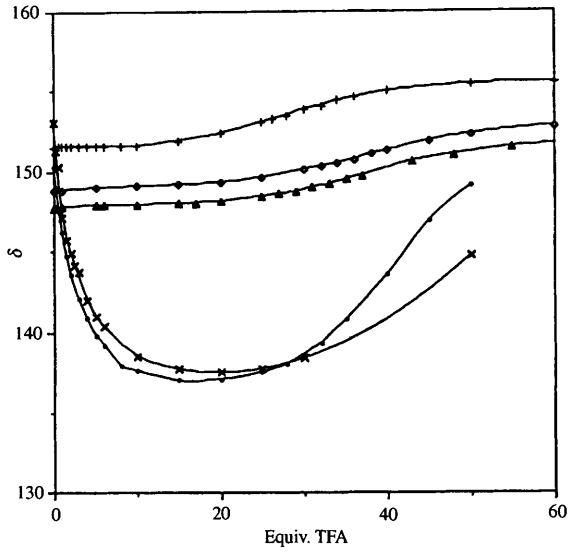


Fig. 2 Plot of chemical shift of C-4′ against concentration of TFA for **1a** (×), **1b** (●), **1c** (▲), **1d** (◆) and **1e** (+)

respectively. All other carbons of the benzothiazole rings of **1c–e** showed chemical shift changes of greater magnitude than seen when forming the dication. This was because for **1a** and **1b** each of the two protonations resulted in small shifts in opposite directions so that the overall shift differences could not reliably be discerned from effects due to the changing solvent.

^{15}N NMR spectroscopy

Table 3 records ^{15}N NMR data for benzothiazole **1a**. The location of charges (shown by chemical shift changes) and attached protons (shown by inversion or partial cancellation when acquired with full ^1H NOE) are clear and in accord with the ^{13}C data above. In the free base, N-3 has no attached protons whilst N-4′ became a negative triplet when the spectrum was acquired with ^1H NOE, the chemical shift of N-4′ at −311.0 is typical of an aniline.[13] When 10 equiv. of TFA were added (conditions which complete the first protonation) the chemical shift change of −14.9 for the exocyclic nitrogen atom showed that it carried a positive charge. The NOE experiment showed that N-3 had no proton attached under these conditions. In neat TFA, N-3 experiences a further shift of −108.4 ppm and the NOE experiment confirmed **4a** as the structure of the dication.

Consideration of pK_as

The failure, under the conditions used, of the exocyclic amino group to protonate in **1c–e** may be accounted for by consideration of the pK_as of substituted anilines. The pK_as of aniline (4.63) and *o*-toluidine (4.44) are comparable but the halogenated anilines *o*-chloroaniline (2.65), *o*-bromoaniline (2.53) and *o*-iodoaniline (2.60) are 100-fold weaker bases.[14,15] Insertion of a *para*-electron withdrawing group lowers the pK_a still further, for example *p*-cyanoaniline (1.74) and *o*-chloro-*p*-nitroaniline (−0.94).[15] The two pK_as for the protonation of **1a** have been measured[4] as 3.0 and 0.1. Compounds **1a,b** would be expected to have equivalent pK_a values. In contrast, in amines **1c–d** the anticipated very low pK_a of the exocyclic amine results in protonation occurring first at N-3 but at a higher pH than that required to effect the formation of a dication.

Conclusions

The ^{13}C chemical shift changes during the first protonation of **1a,b** indicate the shifting of electron density away from the thiazole towards the phenyl ring: this would increase the double bond character of the C-2–C-1′ bond and thus extend the π-system, thereby accounting for the observed colour change. On the second protonation, chemical shift changes showed electron density revert towards the thiazole ring thus reducing the C-2–C-1′ bond order. The mixture of tautomers proposed by Dey and Dogra[4] is difficult to reconcile with the ^{13}C and ^{15}N NMR data above which are consistent with only a single monocation **2a,b** in the DMSO–TFA solvent mixture.

The results presented herein also have potential pharmaceutical significance. At physiological pH (7.4) all the compounds **1a–e** would be uncharged and lipophilic. However, in the acid of the stomach (4 M HCl, pH *ca.* −0.60) all **1a–e** would be monoprotonated and **1a,b** diprotonated.

Experimental

^1H and ^{13}C NMR spectra were acquired on a Bruker ARX250 spectrometer observing ^1H at 250.13 MHz and ^{13}C at 62.9 MHz using standard Bruker pulse programs. ^{15}N NMR spectra were acquired on a Bruker AC250 spectrometer as previously described.[16] ^1H and ^{13}C shifts are quoted downfield of tetramethylsilane, ^{15}N chemical shifts relative to external nitromethane. *J* values are given in Hz. The synthesis of the free bases **1a–e** is described elsewhere.[1] NMR solvents were purchased from GOSS and TFA from Aldrich.

NMR Titrations

Solutions of the benzothiazoles **1a–e** were prepared [0.15 mmol in 0.5 ml of $(CD_3)_2SO$] and titrated with 0 to 100 equiv. of TFA. ^{13}C-CPD spectra were recorded at each step. By titrating in small steps it was possible to trace the shifting of most carbon atoms through the whole range of acid concentrations although at various stages some signals became broad or disappeared. Graphs of chemical shift against TFA concentration were plotted for each carbon atom, the plot of C-4′ for all the drugs is shown in Fig. 2. Some carbon atoms showed typical sigmoidal titration curves from which the chemical shift change on each protonation and the concentration of TFA to elicit 50% protonation were read. For other carbon atoms a general up or downfield trend, clearly different from effects due to the changing solvent, was observed. The data are presented in Table 2.

The change in chemical shift of the $(CD_3)_2SO$ carbon was noted: across the range of TFA concentrations used there was an upfield shift of 4 ppm, which indicates the solvent effect on chemical shifts during the experiment [at 43 equiv. of TFA the solvent was 1 : 1 TFA : $(CD_3)_2SO$]. Chemical shift differences of less than 4 ppm must therefore be interpreted with caution.

Synthesis

General procedure for the preparation of the dihydrochloride salts of the amines. The amine (0.6 g) was dissolved in 60 ml of a 1 : 1 mixture of ethyl acetate and chloroform; a stream of dry hydrogen chloride was passed through the solution for 40 min. A yellow precipitate formed which was collected by filtration, washed thoroughly with ethyl acetate (2 × 50 ml), chloroform (2 × 50 ml) and dried *in vacuo*.

2-(4-*Aminophenyl*)*benzothiazole dihydrochloride*, **1a**·2*HCl*.—Yield 96%, mp 268–270 °C (Found: C, 52.06; H, 4.24; N, 9.14. $C_{13}H_{10}N_2S$·2HCl requires C, 52.18; H, 4.04; N, 9.36%); ν_{max}/cm^{-1} 2785, 2712, 2562, 1601, 1544, 1507, 1442, 1384, 1226, 966, 836, 761, 722, 700; $\delta_H[(CD_3)_2SO]$ 8.10 (1 H, d, *J* 7.3, 4-H), 8.03 (2 H, d, *J* 8.3, 2′,6′-H), 8.00 (1 H, d, *J* 8.2, 7-H), 7.52 (1 H, dt, *J* 1.2 and 7.3, 5-H), 7.43 (1 H, ddd, *J* 1.2, 7.3 and 8.1, 6-H), 7.25–7.23 (2 H, m, 3′,5′-H), 5.71 (br s, NH_3).

2-(4-*Amino*-3-*methylphenyl*)*benzothiazole dihydrochloride*, **1b**·2*HCl*.—Yield 98%, mp 267–269 °C (Found: C, 53.41; H, 4.48; N, 8.80. $C_{14}H_{12}N_2S$·2HCl requires C, 53.63; H, 4.50; N, 8.94%); ν_{max}/cm^{-1} 2567, 1807, 1597, 1503, 1441, 1379, 1165, 903; $\delta_H[(CD_3)_2SO]$ 8.36 (br s, NH_3), 8.15 (1 H, d, *J* 7.8, 4-H), 8.07–7.96 (3 H, m, 2′,5′,6′-H), 7.60–7.44 (3 H, 5, 6, 7-H), 2.48 (3 H, s, CH_3).

2-(4-*Amino*-3-*bromophenyl*)*benzothiazole dihydrochloride*, **1d**·2*HCl*.—Yield 83%, mp 214–216 °C (Found: C, 41.44; H, 2.87; N, 7.65. $C_{13}H_9BrN_2S$·2HCl requires C, 41.30; H, 2.93; N, 7.41%); ν_{max}/cm^{-1} 2778, 2531, 1584, 1495, 1441, 1381, 1246, 1175, 910; $\delta_H[(CD_3)_2SO]$ 8.36–8.06 (m, including d, *J* 2.0, 4, 2′-H, NH_3), 7.95 (1 H, d, *J* 7.9, 7-H), 7.79 (1 H, d, *J* 2.0 and 8.5, 6′-H), 7.53 (1 H, dt, *J* 1.6 and 7.9, 5-H), 7.42 (1 H, dt, *J* 1.5 and 7.9, 6-H), 6.94 (1 H, d, *J* 8.5, 5′-H).

2-(4-*Aminophenyl*)*benzothiazole monohydrochloride*, **1a**·*HCl*.—To a solution of 2-(4-aminophenyl)benzothiazole (0.6 g, 2.62 mmol) in 60 ml of a mixture of ethyl acetate and chloroform (1 : 1) was added dropwise 2.65 ml of 1 M hydrochloric acid at room temperature. The resultant mixture was stirred for 30 min. The product was collected by filtration, washed thoroughly with ethyl acetate (2 × 50 ml), then chloroform (2 × 50 ml) and dried. Yield 86%, mp 265–268 °C (Found: C, 59.24; H, 4.32; N, 10.43. $C_{13}H_{10}N_2S$·HCl requires C, 59.43; H, 4.22; N, 10.66%); ν_{max}/cm^{-1} 2787, 2564, 1601, 1508, 1443, 966; $\delta_H[(CD_3)_2SO]$ 9.73 (br s, NH_3), 8.30–8.19 (4 H, m, including d, *J* 8.6, 4, 7, 2′,6′-H), 7.22–7.57 (4 H, m, including d, *J* 8.6, 5, 6, 3′,5′-H).

Acknowledgements

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