Published in final edited form as:

J Fluor Chem. 2017 August ; 200: 146–152. doi:10.1016/j.jfluchem.2017.06.017.

Synthesis of pH indicators for Cerenkov imaging by electrophilic substitution of bromine by fluorine in an aromatic system

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Abstract

Direct electrophilic fluorination using molecular fluorine gas is used in organic synthesis to create novel fluorine-containing compounds with potential beneficial activity that could not be obtained by nucleophilic substitution. In this paper, we report a novel electrophilic substitution of bromine by fluorine in an aromatic system. The mechanism of this type of fluorination was explored using the reaction between bromothymolsulfonphthalein (Bromothymol Blue) and dilute fluorine gas under acidic conditions. Substitution occurs in the bromine atoms located in the *ortho*-position relative to the hydroxyl group. A similar electrophilic fluorination of thymolsulfonphthalein (Thymol Blue) leads to a substitution of hydrogen atoms in the same position (*ortho* to hydroxyl). NMR spectroscopy was used to confirm the fluorination sites. NMR spectra of thymolsulfonphthalein and its derivatives under basic conditions can be explained by considering the absence of resonance between the two phenolic rings. Both dibromothymol blue and fluorobromothymol blue revealed intermolecular attenuate Cerenkov radiation selectively near their maximum absorbance in a pH dependent manner.

Graphical Abstract

Keywords

electrophilic fluorination; bromine; fluorine; pH indicators; Cerenkov imaging; Thymol Blue; Bromothymol blue

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Introduction

Fluorinated organic compounds are widely used in various fields of biomedical research [1–3]. These disciplines vary from pharmacology [4–6] to molecular imaging [7, 8], the latter of which includes a promising new technique known as Cerenkov imaging [9–12]. Our fluorine-related research is focused on the synthesis of novel potential anticancer drugs [13–18] and the development of ^{18}F -labeled Cerenkov imaging agents [19–23]. Recently, we reported that direct electrophilic fluorination with dilute fluorine gas under acidic conditions is a useful method for labeling of triarylmethane-based pH indicators with the positron emitting isotope ^{18}F [19–21] for *in vivo* pH measurement [22] by a combination of optical Cerenkov imaging and nuclear positron emission tomography (PET). Cerenkov radiation is a multispectral photonic emission arising when beta particles expelled from a decaying nucleus exceed the speed of light in their surrounding medium. The attachment of an ^{18}F isotope to a pH sensitive molecule provides an internal Cerenkov beacon, which is partially absorbed by the indicator. A change in pH results in a change in indicator color, resulting in the absorption of a different bandwidth of the multispectral Cerenkov radiation. This change in absorption can be detected *in vitro* or *in vivo* and provides a method for non-invasive pH measurement.

We have previously shown that electrophilic fluorination of triarylmethane-pH indicators results in a substitution of the hydrogen atom in the *ortho*-position to the hydroxyl group of the phenol or naphthol ring [19–21]. A similar type of fluorination was also observed for compounds containing a phenoxazone ring system [23]. Further investigation of this reaction led to the finding that electrophilic fluorination of triarylmethane molecules containing bromine atoms in the *ortho*-position of the phenol rings results in exchange of bromine by a fluorine atom.

In this paper, we report a new reaction: an electrophilic substitution of bromine by fluorine in an aromatic ring system. This type of reaction is demonstrated by the fluorination of bromothymolsulfonphthalein, commonly known as Bromothymol Blue, but referred to in this paper as dibromothymol blue (DBTB). We used direct fluorination with F_2 to obtain fluorobromothymol blue (FBTB) and difluorothymolsulfonphthalein (difluorothymol blue, DFTB), to be used as pH indicators, and to study the reaction mechanism. To compare the properties of the obtained fluorinated pH indicators and to prove the identity of the disubstituted product (DFTB), we performed a fluorination of the analogous pH indicator lacking the bromine atoms, thymolsulfonphthalein, commonly known as Thymol Blue (TB). The parent compound (DBTB) and a monofluorinated compound (FBTB) were assayed for their ability to selectively absorb intermolecular Cerenkov radiation in a pH dependent fashion and the Cerenkov pK_a s were compared to those measured by absorbance. Detailed analysis by mass spectrometry and NMR spectroscopy was used to identify the mechanism of the fluorine-bromine exchange.

Materials and Methods

All reagents were purchased from Sigma-Aldrich, with the exception of the 0.1% fluorine-neon mixture, which was obtained from Matheson TriGas Inc. (Twinsburg, OH). The reaction was performed by bubbling 0.1% F₂ in Ne for 5 minutes through a solution of the corresponding indicator in acetic acid (2 mg/mL). The reaction mixture was evaporated in a vacuum at 110 °C, redissolved in 2 mL of water/acetonitrile injected into a semi-preparative HPLC column (Phenomenex, Synergi 4 µm Hydro-RP 80 Å 10×250 mm), and eluted by a 30 % acetonitrile-water solvent system for TB and 45% acetonitrile-water solvent system for DBTB at 3 mL/min with detection of absorption at 430 nm. Under these conditions, the elution times for the products of interest were in the range of 20 – 30 min with reasonable separation of initial precursor and fluorination products.

Corresponding fractions were collected and the reaction products analyzed by analytical HPLC, mass-spectrometry, UV-visible spectroscopy, and ¹H and ¹⁹F NMR. The presence of the individual compounds in the fractions was confirmed by analytical HPLC performed with a 4.6 × 250 mm column of the same type using a gradient of acetonitrile-ammonium acetate (0.1 M, buffer pH 4.7). The incorporation of fluorine atoms into the indicator molecule was confirmed on a Thermo Fisher Scientific Orbitrap Exactive mass spectrometer (Bremen, Germany) with Xcalibur software. UV-visible spectroscopic analysis of the absorption and indicator properties of the products was performed on a Molecular Devices SpectraMax M5 (Sunnyvale, CA) spectrophotometer using citrate, phosphate, and borate buffers for different values of pH. The position of the fluorination in the molecules was determined from ¹H and ¹⁹F NMR spectra obtained on a Bruker DMX-360 spectrometer using deuterated water and methanol-d₄ solvent. Decoupling experiments for ¹H {¹⁹F} were carried out with O1 = 4.5 ppm, O2 = -136 ppm.

Cerenkov experiments were performed on a PerkinElmer (Hopkinton, MA) IVIS Lumina II imaging system. Around 100 µCi of ¹⁸F-FDG in 100 µL was added to wells containing 100 µL 100 µM (final concentration 50 µM) DBTB or fluorobromothymol blue (FBTB) at different pH from 4 to 10. Images were obtained using the open filter (measuring all light emitted) and the four filters GFP (515 – 575 nm), DSred (575 – 650 nm), Cy5.5 (695 – 750 nm), and ICG (815 – 875 nm) available on the Lumina II. For these acquisitions, the following parameters were used: each filter was imaged for 60 s, open filter was imaged for 30 s, the stage temperature was set at 37 °C, f-stop = 1 and the field of view = 12.5. Using the Living Image software, ROIs were measured by adjusting an 8 × 12 grid over the plate image; binning factor for analysis was set at 4; the signal at different filters was plotted as a function of pH.

Results

The reaction between DBTB and dilute fluorine gas under acidic conditions results in a substitution of bromine by fluorine. HPLC analysis of the mixture after DBTB fluorination shows the presence of the initial precursor and one major product obtained with 3–4% yield. Mass spectrometry of the purified reaction product reveals two peaks with nearly identical intensity at *m/z* 561.08 and 563.07 (SM Fig. 1). These peaks represent the almost equal

natural abundance of the two bromine isotopes: ^{79}Br (50.7%) and ^{81}Br (49.3%) [24]; indicating the presence of one bromine atom in the molecule, along with the corresponding addition of one atom of ^{19}F . The molecular mass of the compound corresponds to a derivative of bromothymol blue containing one fluorine and only one bromine atom in a molecule, or FBTB. The ^1H and ^{19}F NMR data also support an exchange of bromine by fluorine in the molecule, as will be discussed below. The reaction product also has pH indicator capacity with a pK_a value of 7.56 and an absorption maximum of 610 nm, in the basic form.

A secondary product from the reaction was also purified although in very low yield (<1%). Chemical analysis reveals that this product can also act as a pH indicator with a $\text{pK}_a = 7.65$ and $\lambda_{\text{max}} = 605$ nm (basic pH). We expect this compound to be the product of substitution of the second bromine atom, difluorothymolsulfonphthalein (DFTB). To synthesize this product in quantities sufficient for more detailed characterization, we performed fluorination of TB.

The reaction between TB and dilute fluorine gas in acetic acid is similar to the previously observed fluorination of other triarylmethanes, resulting in fluorine-hydrogen substitution in *ortho*-position of the phenolic rings [19–21]. Separation of the reaction mixture by semi-preparative HPLC indicates the presence of non-reacted TB and two major products. These products have higher retention time on the reverse-phase column, which is typical for more hydrophobic fluorinated derivatives [19–21].

Mass spectrometric analysis of the two HPLC fractions revealed main peaks at m/z 483.17 and 501.16, which correspond to monofluorinated (monofluorothymolsulfonphthalein, MFTB) and difluorinated (DFTB) derivatives of TB. The chemical yield of the products was calculated from the absorption of their basic forms using the extinction coefficient found for TB ($3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) [25]. The yield varied between experiments, around 8–10% for MFTB and 3–4% for DFTB. ^1H and ^{19}F NMR data show introduction of fluorine atoms into the *ortho*-position to the hydroxyl group as will be discussed below.

Both fluorinated derivatives of TB act as pH indicators with properties similar to the parent compound. The yellow neutral forms of the molecules have weak absorption maxima close to 430 nm, while the blue basic forms of the indicators absorbed at 599 nm for MFTB and 605 nm for DFTB. The pK_a values of the fluorinated derivatives were found to be 8.25 for MFTB and 7.65 for DFTB. These results are similar to the behavior of other fluorinated pH indicators [19–21, 23]: successive incorporation of fluorine atoms into the TB molecule ($\text{pK}_a=8.85$, $\lambda_{\text{basic}}=597$ nm) causes a stepwise decrease of the pK_a values and a bathochromic shift of λ_{max} in the basic form. The optical properties of DFTB were identical to the minor product of DBTB fluorination, supporting our hypothesis of fluorine substitution of both bromine atoms.

The reaction scheme of electrophilic fluorination of DBTB and TB is shown in Fig. 1.

The fluorination of TB also resulted in a product with a relatively high yield (5%), which acted as a pH indicator with properties quite different from the initial precursor. This compound had a pK_a equal to 8.6 and absorption maximum of its basic form at 576 nm.

Mass spectrometric analysis of the product revealed a main signal at m/z 384.94, suggesting the loss of sulfonic group (SO_3 , 80 amu) by the parent molecule. Analysis of the literature [25] suggests that this compound might be the indicator thymolbenzein, which has the structure of TB without the sulfonic group and changes its color from yellow at pH 7.6 to blue at pH 9. Since this product does not contain fluorine atoms, it was not used in further experiments. The formation of this product reflects an unusual feature of TB fluorination in comparison with other phenolsulfonphthalein derivatives, where the loss of sulfonic group was never observed [19, 20].

The ability of FBTB to attenuate Cerenkov radiation was determined and compared to the parent compound (Fig. 2). Using an IVIS Lumina II imaging system, we measured the absorption of Cerenkov radiation by determining the attenuation of the signal in response to an increase in pH. In this experiment, we are detecting an intermolecular quenching of the Cerenkov signal between ^{18}F -FDG and the pH indicators. The titration curve in Fig. 2B quantitates the attenuation of the signal in the 575 – 650 nm filter, which encompasses the λ_{max} of the compounds. The pK_a of each compound was determined to be 6.62 and 6.76 for DBTB and FBTB, respectively. The pK_a s determined by Cerenkov radiation are about 0.8 pKa units lower than those observed using UV-vis spectrophotometry, consistent with previous results [22].

A summary of the spectral data and the acid-base properties of the fluorinated derivatives of TB and DBTB is presented in Table 1.

We used ^1H NMR spectroscopy to confirm the introduction of the fluorine atoms into the molecules, as shown by mass spectrometry, and identify the position of substitution (Fig. 3, and detailed in the Experimental section). For all TB derivatives four protons from the ring containing the sulfonic group give four separate signals in the range of δ 7–8 ppm, which were not affected by substitution in the molecule. This indicates that the presence of the sulfonic group in the benzene ring completely deactivates it for electrophilic fluorination with all the substitution reactions occurring in the more active phenolic rings. The same kind of reactivity was observed for the similar molecule cresolsulfonphthalein [20]. It should also be mentioned that in our initial work [19] we presumed a fluorination of the sulfonated ring for phenolsulfonphthalein, which was proven to be not a correct assumption.

The detailed assignment of the proton signals from phenolic rings is presented in Fig. 4, with chemical shifts and coupling constants provided in the Experimental section.

Experimental

Thymolsulfonphthalein (Thymol Blue): ^1H NMR (360 MHz, neutral CD_3OD , δ TSP) 0.85–1.00 (*m*, 12H); 1.81 (*s*, 3H); 1.90 (*s*, 3H); 2.98 (*septet*, $J = 6.8$ Hz, 2H); 6.37 (*s*, 1H); 6.44 (*s*, 1H); 6.94 (*d*, $J = 7.6$ Hz, 1H); 6.98 (*s*, 1H); 7.02 (*s*, 1H); δ 7.38 (*t*, $J = 7.6$ Hz, 1H); 7.44 (*t*, $J = 7.6$ Hz, 1H); 8.04 (*d*, $J = 7.6$ Hz, 1H).

^1H NMR (360 MHz, basic D_2O , δ TSP) 0.95–1.10 (*m*, 12H); 1.74 (*s*, 3H); 1.86 (*s*, 3H); 3.04 (*septet*, $J = 6.8$ Hz, 2H); 6.45 (*s*, 1H); 6.50 (*s*, 1H); 6.80 (*s*, 1H); 6.95 (*s*, 1H); 7.14 (*d*, $J = 7.6$ Hz, 1H); δ 7.53 (*t*, $J = 7.6$ Hz, 1H); 7.67 (*t*, $J = 7.6$ Hz, 1H); 8.04 (*d*, $J = 7.6$ Hz, 1H).

Monofluorothymolsulfonphthalein (MFTB): m/z 483.17; $pK_a = 8.25$, $\lambda_{\text{basic}} = 599$ nm.

^1H NMR (360 MHz, D_2O , δ TSP) 0.85–1.10 (*m*, 12H); 1.68 (*s*, 1.7H); 1.74 (*d*, $J_{\text{HF}} = 3.3$ Hz, 1.3H); 1.78 (*s*, 1.3H); 1.89 (*d*, $J_{\text{HF}} = 3.3$ Hz, 1.7H); 3.14 (*septet*, $J = 6.8$ Hz, 2H); 6.44 (*s*, 0.57H); 6.47 (*s*, 0.43H); 6.58 (*s*, 0.57H); 6.78 (*s*, 0.43H); 6.86 (*s*, 0.43H); 6.96 (*s*, 0.57H); 7.12 (*d*, $J = 7.6$ Hz, 1H); δ 7.50 (*t*, $J = 7.6$ Hz, 1H); 7.67 (*t*, $J = 7.6$ Hz, 1H); 8.04 (*d*, $J = 7.6$ Hz, 1H).

^1H { ^{19}F } decoupling converts doublets δ 1.73 and 1.88 into singlets.

^{19}F (338 MHz, D_2O , δ CFCl_3): -137.33 (*d*, $J_{\text{HF}} = 3.3$ Hz, 0.5F); δ -137.51 (*d*, $J_{\text{HF}} = 3.3$ Hz, 0.5F).

Difluorothymolsulfonphthalein (DFTB): m/z 501.16; $pK_a = 7.65$, $\lambda_{\text{basic}} = 605$ nm.

^1H NMR (360 MHz, D_2O , δ TSP) 0.89–1.06 (*m*, 12H); 1.69 (*d*, $J_{\text{HF}} = 3.0$ Hz, 3H); 1.85 (*d*, $J_{\text{HF}} = 3.0$ Hz, 1.5H); 3.09 (*septet*, $J = 6.8$ Hz, 2H); 6.66 (*s*, 1H); 6.82 (*s*, 1H); 7.11 (*d*, $J = 7.6$ Hz, 1H); δ 7.49 (*t*, $J = 7.6$ Hz, 1H); 7.68 (*t*, $J = 7.6$ Hz, 1H); 8.03 (*d*, $J = 7.6$ Hz, 1H).

^1H { ^{19}F } decoupling converts doublets δ 1.69 and 1.85 into singlets.

^{19}F (338 MHz, D_2O , δ CFCl_3): -135.74 (*d*, $J_{\text{HF}} = 3.0$ Hz, 1F); δ -136.45 (*d*, $J_{\text{HF}} = 3.0$ Hz, 1F).

Fluorobromothymolsulfonphthalein (FBTB): m/z 561.08, 563.07; $pK_a = 7.56$, $\lambda_{\text{basic}} = 610$ nm.

^1H NMR (360 MHz, D_2O , δ TSP) 0.83–1.10 (*m*, 12H); 1.71 (*d*, $J_{\text{HF}} = 3.0$ Hz, 1.6H); 1.82 (*d*, $J_{\text{HF}} = 3.0$ Hz, 1.4H); 2.01 (*s*, 1.4H); 2.09 (*s*, 1.6H); 3.10 (*septet*, $J = 6.8$ Hz, 2H); 6.71 (*s*, 0.45H); 6.74 (*s*, 0.55H); 6.77 (*s*, 0.55H); 6.86 (*s*, 0.45H); 7.16 (*d*, $J = 7.6$ Hz, 1H); δ 7.56 (*t*, $J = 7.6$ Hz, 1H); 7.71 (*t*, $J = 7.6$ Hz, 1H); 8.07 (*d*, $J = 7.6$ Hz, 1H).

^{19}F (338 MHz, D_2O , δ CFCl_3): -135.30 (*d*, $J_{\text{HF}} = 3.0$ Hz, 0.5F); δ -136.20 (*d*, $J_{\text{HF}} = 3.0$ Hz, 0.5F).

Discussion

Nucleophilic substitution of an aliphatic bromine by a fluoride ion in non-aqueous media is one of the well-known methods for labeling molecules with an ^{18}F isotope. This reaction is not frequently used because bromine is not considered a good leaving group and a tosylate or triflate is usually preferable. Still, it can be suitable for molecules containing other non-protected nucleophilic groups to minimize intermolecular reactions [26].

While direct electrophilic fluorination in phenol-type molecules with H-F exchange is well known [27–39], similar Br-F exchange in aromatic molecules has not been previously described. For organic compounds, to the best of our knowledge, the only electrophilic substitution of bromine with fluorine was described for 1- or 2-bromoadamantanes [40].

This new type of electrophilic fluorine substitution of an aromatic ring can be used to introduce an ^{18}F label into complex organic molecules.

The suggested mechanisms of H-F and Br-F electrophilic substitutions in TB and DBTB are presented in Fig. 5. The fluorination of TB with F_2 in HOAc includes direct attack by F_2 (Fig. 5i) or/and intermediately formed hypofluorite FOAc (Fig. 5ii) that was suggested by Chambers, et al. [30]. For electrophilic Br-F substitution in DBTB the mechanism can be similar to the suggestion by Rozen, et al. [40]: it includes fluorination of bromine atom followed by substitutive elimination of BF_3 (Fig. 5iii). Alternative mechanisms might include single electron transfer steps [40] or catalysis by bromine [41] (not shown).

The substitution of Br by F was observed with mass spectrometry and confirmed with ^1H and ^{19}F NMR spectroscopy.

Introduction of fluorine atoms into the triarylmethane molecule modified its optical and acid-base properties in a predictable manner, which we have reported in previous publications [19–23]. Each fluorine atom added to the TB molecule decreases the pK_a value by 0.60 pH units (from 8.85 for TB to 8.25 for MFTB and 7.65 for DFTB). The corresponding pK_a decrease by the introduction of one bromine atom can be estimated as -0.74 (half the difference between 8.85 for TB and 7.37 for DBTB). The predicted pK_a value for FBTB is therefore $8.85 - 0.60 - 0.74 = 7.51$, which is very close to the experimentally observed 7.56. The same calculations are applicable for the bathochromic shift of the wavelength of the absorption maximum in basic conditions of double substituted TB derivatives. Two fluorine atoms in the molecule increase the λ_{max} in basic form by 8 nm (+4 nm per atom), and two bromine atoms by 18 nm (+9 nm per atom). The effect of the simultaneous presence of bromine and fluorine atoms in the molecule is equal to +13 nm, which is equal to the sum of the effects of both atoms in double substituted TB derivatives.

Our purpose in designing fluorine labeled pH indicators is to use the radioactive ^{18}F -labeled analogs to non-invasively measure pH *in vivo* using Cerenkov radiation [22]. With a molecular switching pH indicator, like the ones reported above, we could selectively absorb photons from the radioactive emission in a pH dependent fashion. In this paper, we have demonstrated the intermolecular attenuation of Cerenkov radiation by mixing ^{18}F -FDG with the pH indicators. This was performed only for the brominated derivatives, which have near-physiological pK_a s and allow a comparison with the parent compound. The use of these indicators for cell or *in vivo* work would require the production of the ^{18}F isotopomers, which would only be possible for compounds containing fluorine. It is of interest to note that the pK_a determined using Cerenkov attenuation is about 0.8 pH units lower than that determined using spectrophotometry, consistent with our previous observations [22].

Analysis of the ^1H and ^{19}F NMR spectra for phenolic rings demonstrates one unusual feature for all the TB derivatives. The spectrum in Fig. 3a represents a solution of the yellow form of TB in CD_3OD , where it exists as single charged anion shown in Fig. 4a. This form contains two non-equivalent phenol rings (phenolic and quinoid), which results in pairs of signals from aromatic methyl groups (δ 1.81 and 1.9) and two different aromatic protons (δ 6.37 and 6.44; 6.94 and 6.98). All the NMR modeling programs (ChemDraw Professional, v.

15.0.0.106, MestReNova v.6.0.2-5475) suggest a shift into higher field for *ortho*- protons in the quinoid ring and for *meta*-protons in the phenolic ring, therefore we made a corresponding assignment of the signals on Fig. 4a. The shift of the methine groups in isopropyl moiety is mostly affected by their neighboring methyl groups; therefore, they both result in only one septet (δ 2.98) without being affected by fluorination of aromatic ring. The spectra of the aliphatic methyl groups demonstrate an unusual behavior: instead of the expected doublet from twelve identical protons we observe a complicated set of peaks. This is most likely associated with limited rotation due to the bulky isopropyl groups and the existence of relatively stable non planar “propeller” rotamers [42, 43]; thus, we can consider the signals of these methyl groups to be a combination of doublets at δ 1 ppm as can be seen in Fig. 3a.

Under basic conditions, TB forms a doubly charged anion, which is presumed to be a hybrid of two resonance structures (Fig. 4b) with two equivalent phenolic rings. This resonance suggests the same shift of NMR signals for analogous proton from both rings. However, for doubly charged TB anion we observed a more complicated NMR spectrum (Fig. 3b), which is similar to the spectrum of the single charge anion (Fig. 3a). It also contains a pair of signals for aromatic methyl groups (δ 1.74 and 1.86), two pairs of signals from hydrogen atoms (δ 6.45 and 6.50; 6.80 and 6.95), one signal from methine group (δ 3.04), and a complicated set of doublets for aliphatic methyl groups. This similarity of the spectra suggests a similarity between the structures of the single and double charged TB anions. This means that the basic form of TB also contains two non-equivalent phenol rings, as was previously mentioned by Balderas-Hernandez, et al. [44] without further explanation. As a result, the resonance hybridization between the two structures, shown in Fig. 4b, does not occur for the double charged TB anion. Instead we suggest that the structure of the double charged TB anion has two non-equivalent phenolic rings, as shown in Fig. 4c. This observation is quite surprising, especially considering the fact that other similar triarylmethane derivatives do not demonstrate any peculiarity of this kind [20]. Since the only difference between Cresol Purple [20] and TB is the presence of the isopropyl group on the phenolic rings, the absence of the resonance in the case of TB can be explained by steric factors. Two bulky isopropyl substituents in adjacent rings cause distortion of the molecule, and the phenolic rings become non-coplanar forming a propeller-like structure [42, 43].

Introduction of the fluorine atom into TB molecule makes it even less symmetrical, and therefore the molecule of MFTB exists as two isomers, shown in Fig 4d. This isomerism results in further complication of its ^1H NMR spectra, causing doubling of the proton signals from the phenolic ring. As a result we can observe four signals from the methyl groups and six signals from the phenolic protons (Fig 3c). Two signals from the methyl groups become doublets due to coupling with fluorine atom through three carbons in the aromatic system, suggesting its location in the *ortho*-position to the hydroxyl group/oxygen atom. Analysis of the phenolic proton signals shows that for non-substituted rings the position of the signals is very close to values in TB, changing correspondingly from δ 6.45, 6.50, 6.80, and 6.95 into δ 6.44, 6.47, 6.78 and 6.96. (Actually, the spectrum on Fig. 2c was made from a sample of MFTB with $\sim 1/6$ impurity of TB, and its small peaks are visible adjacent to higher peaks of MFTB.) Substitution of hydrogen by fluorine in the *ortho*-position eliminates two corresponding proton signals and causes a shift of the other two protons in the same ring

into higher field (from δ 6.80 to 6.58 in the phenolic ring, and from δ 6.95 to 6.78 in the quinoid ring). Previously we observed a similar shift into stronger field for proton signals in the *para*-position to the fluorine atom for substituted phenolphthalein [21]. These ^1H NMR data confirm the substitution of the hydrogen atom in *ortho*-position to phenolic hydroxyl, as it is shown in Fig. 1.

The intensities of the ^1H NMR signals for two double charged anion isomers of MFTB are not absolutely equal, suggesting a shift of the equilibrium toward one of the forms. Comparison of experimental data shows that the isomer with the fluorine atom in phenolic ring is slightly more preferable with $K_{\text{eq}} = 1.3$, as it is indicated in Fig. 4d.

Substitution of two protons by fluorine in DFTB makes the molecule symmetrical again (Fig 4e), therefore we can observe only two signals from the aromatic methyl groups (δ 1.69 and 1.85; both are coupled with fluorine) and two aromatic protons from phenol rings (δ 6.66 and 6.82), as it is seen in Fig. 3d.

The NMR spectrum of FBTB confirms the substitution of bromine by fluorine during DBTB fluorination. Since the molecule is asymmetric, it exists as two isomers shown in Fig 4f. As expected from these structures, we observe four signals for aromatic methyl groups (two of them are coupled with fluorine), and four signals from aromatic protons (Fig. 3e), which are assigned to corresponding protons in Fig. 4f. Two forms of the FBTB double charged anion are also not equivalent with a slight ($K_{\text{eq}} = 1.2$) shift to the form with bromine atom located in the phenolic ring.

^{19}F NMR spectra confirm the proposed structure of the fluorinated derivatives of TB and presence of non-equivalent phenolic rings without resonance in their molecules. All three fluorinated products have two ^{19}F signals in the range of δ -135 –140 ppm, which are coupled with protons from the adjacent methyl aromatic group. This chemical shift is specific for *ortho*-substituted phenols [45] and together with coupling on methyl protons is a confirmation of fluorine incorporation into this position. The presence of two different fluorine signals in all the molecules suggests existence of two different phenol rings, proving lack of resonance in the double charged anion of TB and its derivatives.

Conclusion

We observed a novel reaction of electrophilic substitution of bromine by fluorine in aromatic systems. The reaction occurs under acidic conditions between diluted fluorine gas and aromatic molecules having phenol system with bromine atoms in *ortho*-position to hydroxyl groups. This novel reaction can be used for the introduction of an ^{18}F -label into complex organic molecules.

The analysis of ^1H and ^{19}F NMR spectra of double negative charged anions of Thymol Blue and its derivatives suggests an unusual feature of these molecules: the presence of two non-equivalent phenolic rings. This result can be explained by the absence of resonance in the system of two non-coplanar phenolic rings due to distortion of the structure by two bulky substituent isopropyl groups. Fluorobromothymol blue was shown to act as a pH-indicator and selectively attenuate the Cerenkov radiation emitted by ^{18}F -FDG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by R01 EB018645 (EJD), T32 GM008076 (ADA) F31 CA206453-01 (ADA). The authors thank Benjamin K. Liebov, Ph.D., for discussion.

Literature

1. Bégue, JP., Bonnet-Delpon, D. Bioorganic and Medicinal Chemistry of Fluorine. Wiley, Hoboken: 2008.
2. Gouverneur, V., Müller, K. Fluorine in Pharmaceutical and Medicinal Chemistry: From Biophysical Aspects to Clinical Applications. Imperial College Press; London: 2012.
3. Ojima, I. Fluorine in Medicinal Chemistry and Chemical Biology. Wiley, Chichester: 2009.
4. Zhou Y, Wang J, Gu Z, Wang S, Zhu W, Acena JL, Soloshonok VA, Izawa K, Liu H. Next Generation of Fluorine-Containing Pharmaceuticals, Compounds Currently in Phase II–III Clinical Trials of Major Pharmaceutical Companies: New Structural Trends and Therapeutic Areas. Chem. Rev. (Washington, D, U. S.). 2016; 116(2):422–518.
5. Zhu W, Wang J, Wang S, Gu Z, Acena JL, Izawa K, Liu H, Soloshonok VA. Recent advances in the trifluoromethylation methodology and new CF₃-containing drugs. J. Fluor. Chem. 2014; 167:37–54.
6. Wang J, Sanchez-Rosello M, Acena JL, del Pozo C, Soroichinsky AE, Fustero S, Soloshonok VA, Liu H. Fluorine in Pharmaceutical Industry: Fluorine-Containing Drugs Introduced to the Market in the Last Decade (2001–2011). Chem. Rev. (Washington, DC, U. S.). 2014; 114(4):2432–2506.
7. Weissleder, R. Molecular Imaging: Principles and Practice. People's Medical Publishing House; Shelton: 2010.
8. Pomper, MG., Gelovani, JG. Molecular Imaging in Oncology. Taylor & Francis, New York: 2008.
9. Ciarrocchi E, Belcari N. Cerenkov luminescence imaging: physics principles and potential applications in biomedical sciences. EJNMMI Phys. 2017; 4(1):14. [PubMed: 28283990]
10. Grootendorst MR, Cariati M, Purushotham A, Kothari A, Tuch DS. Cerenkov luminescence imaging (CLI) for image-guided cancer surgery. Clin Transl Imaging. 2016; 4(5):353–366. [PubMed: 27738626]
11. Spinelli AE, Boschi F. Novel biomedical applications of Cerenkov radiation and radioluminescence imaging. Phys Med. 2015; 31(2):120–9. [PubMed: 25555905]
12. Lucignani G. Cerenkov radioactive optical imaging: a promising new strategy. Eur J Nucl Med Mol Imaging. 2011; 38(3):592–5. [PubMed: 21174087]
13. Luzina EL, Popov AV. Synthesis and anticancer activity of N-bis(trifluoromethyl)alkyl-N'-thiazolyl and N-bis(trifluoromethyl)alkyl-N'-benzothiazolyl ureas. Eur. J. Med. Chem. 2009; 44(12):4944–4953. [PubMed: 19740574]
14. Luzina EL, Popov AV. Anticancer activity of N-bis(trifluoromethyl)alkyl-N'-(polychlorophenyl) and N'-(1,2,4-triazolyl) ureas. Eur. J. Med. Chem. 2010; 45(11):5507–5512. [PubMed: 20850204]
15. Luzina EL, Popov AV. Synthesis, evaluation of anticancer activity and COMPARE analysis of N-bis(trifluoromethyl)alkyl-N'-substituted ureas with pharmacophoric moieties. Eur. J. Med. Chem. 2012; 53:364–373. [PubMed: 22538016]
16. Luzina EL, Popov AV. Synthesis of 1-aryl(1-arylsulfonyl)-4-bis(trifluoromethyl)alkyl semicarbazides as potential physiologically active compounds. J. Fluor. Chem. 2013; 148:41–48.
17. Luzina EL, Popov AV. Synthesis and anticancer activity evaluation of 3,4-mono- and bicyclosubstituted N-(het)aryl trifluoromethyl succinimides. J. Fluor. Chem. 2014; 168:121–127. [PubMed: 25400294]
18. Luzina EL, Popov AV. Synthesis of 3,3,3-trifluoroethyl isocyanate, carbamate and ureas. Anticancer activity evaluation of N-(3,3,3-trifluoroethyl)-N'-substituted ureas. J. Fluor. Chem. 2015; 176:82–88. [PubMed: 26213416]

19. Kachur AV, Popov AV, Karp JS, Delikatny EJ. Direct Fluorination of Phenolsulfonphthalein: A Method for Synthesis of Positron-Emitting Indicators for In Vivo pH Measurement. *Cell Biochem. Biophys.* 2013; 66(1):1–5. [PubMed: 22790882]
20. Kachur AV, Sardelis D, Bentzley C, Popov AV, Delikatny EJ, Karp J. Synthesis and characterization of fluorinated derivatives of cresolsulfonphthalein. *J. Fluor. Chem.* 2013; 145:112–117. [PubMed: 25411509]
21. Kachur AV, Popov AA, Delikatny EJ, Karp JS, Popov AV. Synthesis of 18F-labeled phenolphthalein and naphtholphthalein. *J. Fluor. Chem.* 2013; 151:1–6. [PubMed: 25411510]
22. Czupryna J, Kachur AV, Blankemeyer E, Popov AV, Arroyo AD, Karp JS, Delikatny EJ. Cerenkov-specific contrast agents for detection of pH in vivo. *J. Nucl. Med.* 2015; 56(3):483–488. [PubMed: 25655631]
23. Kachur AV, Arroyo AD, Popov AV, Saylor SJ, Delikatny EJ. Synthesis of F-18 labeled resazurin by direct electrophilic fluorination. *J. Fluor. Chem.* 2015; 178:136–141. [PubMed: 26504251]
24. Dean, JA. *Lange's Handbook of Chemistry*. 13. McGraw-Hill; New York: 1985.
25. Sabnis, RW. *Handbook of Acid-Base Indicators*. CRC Press; Boca Raton: 2007.
26. Kachur AV, Dolbier WR Jr, Evans SM, Shiue C-Y, Shiue GG, Skov KA, Baird IR, James BR, Li A-R, Roche A, Koch CJ. Synthesis of new hypoxia markers EF1 and [18F]-EF1. *Appl. Radiat. Isot.* 1999; 51(6):643–650. [PubMed: 10581679]
27. Misaki S. Direct fluorination of phenol and cresols. *J. Fluor. Chem.* 1981; 17(2):159–71.
28. Chambers RD, Fox MA, Sandford G, Trmcic J, Goeta A. Elemental fluorine. Part 20. Direct fluorination of deactivated aromatic systems using microreactor techniques. *J. Fluor. Chem.* 2006; 128(1):29–33.
29. Chambers RD, Hutchinson J, Sparrowhawk ME, Sandford G, Moilliet JS, Thomson J. Elemental fluorine Part 12. Fluorination of 1,4-disubstituted aromatic compounds. *J. Fluor. Chem.* 2000; 102(1–2):169–173.
30. Chambers RD, Skinner CJ, Thomson J, Hutchinson J. Electrophilic fluorination using elemental fluorine. *J. Chem. Soc., Chem. Commun.* 1995; (1):17.
31. Chirakal R, Brown KL, Firnau G, Garnett ES, Hughes DW, Sayer BG, Smith RW. Synthesis of 2- and 3-fluorotyrosine with dilute fluorine gas. *J. Fluor. Chem.* 1987; 37(2):267–78.
32. Sandford G. Product class 1: fluoroarenes. *Sci. Synth.* 2007; 31a:21–78.
33. Hessel V, Loeb P, Loewe H. Direct fluorination of aromatics with elemental fluorine in microstructured reactors. *Chim. Oggi.* 2004; 22(6):10–12. 14–15.
34. de Mas N, Guenther A, Schmidt MA, Jensen KF. Microfabricated Multiphase Reactors for the Selective Direct Fluorination of Aromatics. *Ind. Eng. Chem. Res.* 2003; 42(4):698–710.
35. Vasdev N, Chirakal R, Schrobilgen GJ, Nahmias C. Selectivity of elemental fluorine towards L-tyrosine and L- α -methyltyrosine in acidic media and the syntheses of their [18F]3-fluoro and [18F]3,5-difluoro derivatives. *J. Fluor. Chem.* 2001; 111(1):17–25.
36. Moilliet JS. The use of elemental fluorine for selective direct fluorinations. *J. Fluor. Chem.* 2001; 109(1):13–17.
37. Moilliet JS. The use of elemental fluorine for selective direct fluorinations. *Chemical & Polymer.* 1999;21/1–21/9.
38. Chambers RD, Hutchinson J, Sandford G. Recent studies at Durham on direct fluorination. *J. Fluor. Chem.* 1999; 100(1–2):63–73.
39. Hutchinson J, Sandford G. Elemental fluorine in organic chemistry. *Top. Curr. Chem.* 1997; 193:1–43. (Organofluorine Chemistry).
40. Rozen S, Brand M. Electrophilic attack of elemental fluorine on organic halogens. Synthesis of fluoroadamantanes. *J. Org. Chem.* 1981; 46(4):733–6.
41. Kachur AV, Dolbier WR Jr, Xu W, Koch CJ. Catalysis of fluorine addition to double bond: An improvement of method for synthesis of 18F PET agents. *Appl. Radiat. Isot.* 2009; 68(2):293–296. [PubMed: 19897377]
42. Santillan R, Farfan N, Castillo D, Gutierrez A, Hopfl H. 13C NMR and crystallographic study of thymolsulfonephthalein. *Chem. - Eur. J.* 1998; 4(10):1904–1909.

43. Schuster II, Colter AK, Kurland RJ. Magnetic resonance studies of triphenylcarbonium ions. I. Fluorine-19 nuclear magnetic resonance studies of conformational equilibria and interconversion. *J. Amer. Chem. Soc.* 1968; 90(17):4679–87.
44. Balderas-Hernandez P, Ramirez MT, Rojas-Hernandez A, Gutierrez A. Determination of pKa's for thymol blue in aqueous medium: evidence of dimer formation. *Talanta*. 1998; 46(6):1439–1452. [PubMed: 18967274]
45. Dolbier, WR. *Guide to Fluorine NMR for Organic Chemists*. Wiley, Hoboken: 2009.

Highlights

- The Br-F electrophilic substitution in an aromatic ring was achieved with diluted F₂ under acidic conditions
- Direct fluorination of thymol (and dibromothymol) blue leads to mono- and difluorinated products
- Substitution occurs in the *ortho*-positions to the hydroxyl group
- Dibromothymol (and bromofluorothymol) blue are pH sensitive absorbing Cerenkov radiation differently in acidic and basic pH
- NMR spectra show absence of resonance in thymolsulfonphthalein anion

Figure 1. Reactions of electrophilic substitution of bromine by fluorine in the molecule of bromothymolsulfonphthalein (DBTB) [top] and electrophilic fluorination of thymolsulfonphthalein (TB) [bottom].

Figure 2.

Intermolecular quenching of Cerenkov radiation by DBTB and FBTB. A. 100 μ L aliquots of DBTB or FBTB were placed in a 96-well plate in pH buffers from pH 4 to pH 10. Around 100 μ Ci of ^{18}F -FDG were added to each well and Cerenkov radiation was detected using the filters in the IVIS Lumina II imaging system. Photographs of the wells are also presented to show the pH dependent color changes. B. pH-dependent attenuation of Cerenkov radiation. The titration curve (from pH 4 to pH 10) shows quantitation of Cerenkov radiation attenuated by the pH indicators in the 575–650 nm filter in the IVIS Lumina II.

Figure 3.

^1H NMR spectra of TB and its derivatives: a) TB in CD_3OD (exists as a single charged anion); b) TB in basic (K_2CO_3) D_2O (a double charged anion); c) MFTB in basic D_2O (the product contains $\sim 1/6$ impurity of TB); d) DFTB; and e) FBTB (both in basic D_2O).

Figure 4.

Structure and assignment of ^1H NMR signals for different forms of TB derivatives. A single charged TB anion (a) existing in CD_3OD has two different phenolic rings, which is reflected in different chemical shifts of the proton signals in phenolic (left) and quinoid (right) rings; the numbers indicate the chemical shift of the correspondent signals for spectrum presented in Fig. 3a. A double charged TB anion is supposed to have two equal rings due to electron delocalization between two resonance structures (b). In reality, we observe a ^1H NMR spectrum (Fig. 3b) similar to the single charged anion, which suggests existence of two different phenolic rings and no resonance, as is shown for the structure (c). For the asymmetrical monofluorinated molecule we observe further complication of the spectrum in Fig. 3c due to the existence of two different isomers of anion shown in (d). The structure of symmetrical DFTB is shown in (e), and two isomers of FBTD are shown in (f).

Figure 5.

Putative mechanisms of electrophilic fluorination in TB and DBTB by F_2 in HOAc: i) direct electrophilic fluorination of H with F in TB by F_2 [30]; ii) electrophilic fluorination of TB by FOAc formed *in situ* [30]; iii) electrophilic substitution of Br with F in DBTB via fluorination of Br followed by BrF_3 elimination (based on [40]).

Table 1

Acid-base properties and absorption of the basic form of Thymol Blue-based pH indicators.

Indicator	pK _a (pK _a)	λ_{max} (λ_{max}) in basic form	pKa (Cerenkov)
TB	8.85	597	N.D.
MFTB	8.25 (−0.6)	599 (+2)	N.D.
DFTB	7.65 (−1.2)	605 (+8)	N.D.
FBTB	7.56 (−1.29)	610 (+13)	6.77
DBTB	7.37 (−1.48)	615 (+18)	6.62

Numbers in parenthesis represent the change in values with respect to TB.

N.D.: not determined.