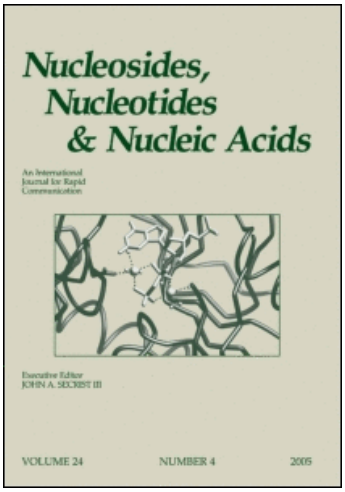

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S. Takatori[a]; A. Matsuda[b]; J. -I. Yamashita[c]; H. Hayatsu[a]; Y. Wataya[a]
[a] Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama, Japan [b] Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan [c] Taiho Pharmaceutical Co., Saitama, Japan

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RAPID, STEPWISE SUBSTITUTION OF FLUORINES IN 5-TRIFLUOROMETHYL-2'-DEOXYURIDINE BY BISULFITE

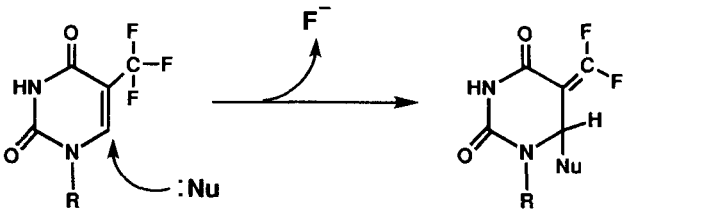
Satoshi TAKATORI, Akira MATSUDA[a], Jun-ichi YAMASHITA[b], Hikoya HAYATSU and Yusuke WATAYA

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan, [a]Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan, and [b]Taiho Pharmaceutical Co., Hanno, Saitama 357, Japan

ABSTRACT: On treatment with bisulfite at neutral pH, 5-trifluoromethyl-2'-deoxyuridine (CF_3dUR) underwent rapid substitution of the fluorine atoms by bisulfite to give first the monosulfonate and then the disulfonate derivatives. It was shown that the monosulfonate product has reactivity to bisulfite with a potency half that of CF_3dUR. These findings demonstrate the stepwise nature of the fluorine release from CF_3dUR and constitute evidence that 5-exo-methylene type intermediates are involved in the nucleophile-mediated release of the fluorine from CF_3dUR.

5-Trifluoromethyl-2'-deoxyuridine 5'-phosphate (CF_3dUMP) is a potent inhibitor against thymidylate synthase, a key enzyme for cellular DNA synthesis.[1] This inhibition seems to be a result of formation of an inactive covalent adduct between CF_3dUMP and the enzyme.[1,2,3] The precise structure of the adduct, however, is yet to be elucidated.

The carbon-fluorine linkages in a CF_3 residue are generally regarded as stable. Examples relevant to the present study are found in the resistance of 6-trifluoromethyluracil,[4] 2-trifluoromethyl-4-oxopyrimidines,[5] and 5-trifluoromethyl-6-azauracil[6] towards hydrolytic reactions. In contrast, the CF_3 residues in 5-trifluoromethyluracil and its nucleosides are readily converted into 5-carboxyuracils in basic media.[7-9] 5-Trifluoromethyluracil reacts with amines in aqueous solutions to give carbamoyluracils.[7] 5-Trifluoromethyl-2'-deoxyuridine (CF_3dUR) reacts with methoxyamine to give a 5-C(=N-OCH_3)N$HOCH_3$ derivative.[3] To explain the reactivity of the C-F linkage, addition of a nucleophile to position 6 of the 5,6-double bond of pyrimidine ring accompanied by cleavage of the C-F bond has been proposed.[2,3,10] According to this



CF_3dUR

R = 2'-deoxy-β-D-ribofuranosyl
Nu = nucleophile

FIG. 1. Mechanism of fluorine-release from CF_3dUR.[2,3,10]

mechanism, the aforementioned reactions of 5-trifluoromethyluracils would involve intermediary formation of corresponding 6-substituted 5-difluoromethylene-5,6-dihydrouracils (FIG. 1).

To gain more knowledge about the reactivity of the CF_3 group, we have investigated the reaction of CF_3dUR with bisulfite, a strong nucleophile well known to add across the 5,6-double bond of uracil and cytosine.[11,12] We wish to describe here that the fluorines in CF_3dUR undergo stepwise substitution with bisulfite: rapid substitutions take place for the first and second fluorine atoms, giving sulfonates. The results also constitute evidence for the formation of 5-exo-methylene type intermediates.

MATERIALS AND METHODS

CF_3dUR was a product of Sigma (St. Louis, U.S.A.), the purity of which was higher than 99% as checked by an HPLC analysis. Bisulfite solutions were prepared fresh from reagent grade sodium hydrogen sulfite (Wako, Tokyo) and sodium sulfite (Wako); the desired pH value was obtained by mixing appropriate amounts of these two agents.

HPLC was performed with a Waters system (model 510) on a reverse phase column of Inertsil ODS (4.6 x 250 mm). The elution was done with 5 mM triethylammonium bicarbonate, pH 6.1, with a linear gradient of methanol in the eluent from 0 to 35 % during a period of 60 min. The flow rate was 0.5 ml per min, and the detection was by absorbance at 254 nm and 280 nm. For isolation of products, preparative HPLC was carried out with a larger column (Inertsil Prep-ODS, 20 x 250 mm). Also, for isolation purpose, paper chromatography was performed on Whatman 3MM filter paper ascendingly, with *n*-butanol-acetic acid-water (2 : 1 : 1, v/v) as a developing solvent.

Paper electrophoresis was done on Toyo Filter Paper No. 51. The buffers used were 0.1 M sodium acetate at pH 4.0 and 0.1 M sodium phosphate at pH 7.0. For reference compounds, uridine 5'-phosphate (UMP) and uridine were used, and the migration distance of a given compound is represented by M_{UMP}, with the UMP migration from uridine being taken as 1.0.

^1H-NMR of compounds in D_2O at 400 MHz was measured on a Jeol JNM-EX400 with sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. ^{19}F-NMR of compounds in CD_3OD at 254 MHz was measured on a Jeol JNM-EX270 with $CFCl_3$ as an internal standard. Fast atom bombardment mass spectra were recorded with a Jeol JMS-HX110 at an ionizing voltage of 70 eV.

The rates of reactions between nucleosides and bisulfite were determined by measuring the decrease in the amounts of the starting nucleosides as analyzed by HPLC.

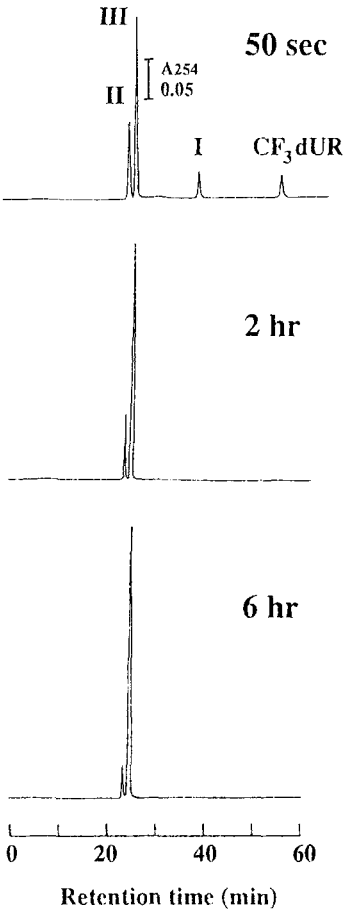


FIG. 2. HPLC profiles of the reaction between CF_3dUR and bisulfite.
CF_3dUR (0.034 M) was treated with sodium bisulfite (1 M) at pH 7.0 and 22°C.

RESULTS

Products in the reaction between CF_3dUR and bisulfite. CF_3dUR (0.034 M) was treated with 1 M sodium bisulfite at pH 7.0 and at 22°C, and the reaction was analyzed by HPLC. As FIG. 2 shows, immediately after the start (50 sec), a major portion of the CF_3dUR disappeared and at least three products (I-III) were formed. At 2 hr of the reaction, product I was no longer present, and at 6 hr the peak corresponding to product II decreased and the peak for product III increased. The A_{254}/A_{280} values for the peak of product II stayed unchanged (0.77) during this 6 hr period, whereas the A_{254}/A_{280} for the peak of product III underwent a large change: 5.64 at 50 sec, 3.01 at 2 hr, and 2.19 at 6 hr. This observation suggested that product II was a single compound, while III was a mixture of compounds. We isolated products I and II, the initial products of this reaction, and determined their structures as 5-difluoro-

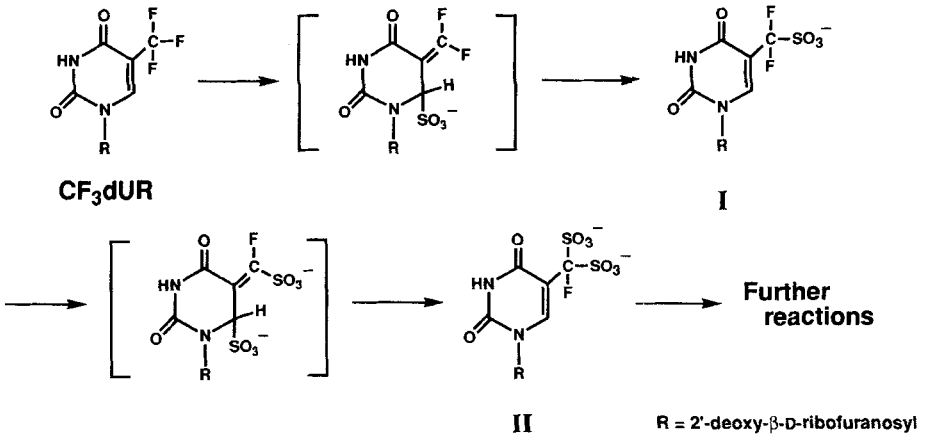


FIG. 3. Course of the reaction between CF_3dUR and bisulfite.

sulfonatomethyl-2'-deoxyuridine (I) and 5-fluorodisulfonatomethyl-2'-deoxyuridine (II), respectively (FIG. 3). The nature of product(s) III was not pursued in the present study.

5-Difluorosulfonatomethyl-2'-deoxyuridine (I). CF_3dUR (0.17 M) was incubated in 0.5 M sodium bisulfite at pH 7.0 and at 22°C for 2 hr. Product I was isolated from the reaction mixture by paper chromatography (R_F 0.30, with CF_3dUR showing an R_F 0.82), followed by repeated preparative HPLC. Product I was obtained as a triethylammonium salt and its physical properties were as follows: ^{1}H-NMR, δ (ppm) 2.44 (m, 2H, 2'-H), 3.81 (octet, 2H, 5'-H), 4.07 (m, 1H, 4'-H), 4.49 (m, 1H, 3'-H), 6.26 (t, 1H, 1'-H), 8.42 (s, 1H, 6-H); ^{19}F-NMR (ppm, upfield from $CFCl_3$) 99.7 (d), 101.8 (d); mass *m/z* 357 $[M - H]^-$; UV (nm), λ_{max} 208, 266 and λ_{min} 232 at pH 6 and pH 2 (ε_{266} at pH 6, 10700), λ_{max} 264 and λ_{min} 239 at pH 13. In paper electrophoresis, product I behaved as a monoanion both at pH 4 and pH 7 (M_{UMP} 1.1 at pH 4, and 0.84 at pH 7).

These results established the structure of product I as 5-difluorosulfonatomethyl-2'-deoxyuridine. The slower movement of the compound in paper chromatography relative to that of CF_3dUR is consistent with the presence of a sulfonate group.

5-Fluorodisulfonatomethyl-2'-deoxyuridine (II). The reaction mixture used for isolating product I contained also product II. In the paper chromatography, product II migrated to R_F 0.11. Material obtained from this zone was fractionated with repeated HPLC, and the purified sample of II obtained as a di-triethylammonium salt showed the following properties: ^{1}H-NMR, δ (ppm) 2.40 (m, 2H, 2'-H), 3.78 (octet, 2H, 5'-H), 4.06 (m, 1H, 4'-H), 4.39 (m, 1H, 3'-H), 6.29 (t, 1H, 1'-H), 8.56 (s, 1H, 6-H); ^{19}F-NMR (ppm,

upfield from $CFCl_3$) 157.1; mass *m/z* 419 $[M - H]^-$; UV (nm), λ_{max} 213, 271 and λ_{min} 237 at pH 6 and pH 2 (ε_{271} at pH 6, 9450), λ_{max} 268 and λ_{min} 244 at pH 13. In paper electrophoresis, product II behaved as having two anionic charges both at pH 4 and pH 7 (M_{UMP} 1.85 at pH 4, and 0.97 at pH 7).

These observations established the structure of II as 5-fluorodisulfonatomethyl-2'-deoxyuridine. The fact that this compound migrates in paper chromatography more slowly than the monosulfonate I is consistent with the disulfonate structure.

Rates of disappearance of CF_3dUR and product I in the reactions with bisulfite. CF_3dUR (0.05 mM) and product I (0.05 mM) were individually treated with 0.1 M sodium bisulfite at pH 7.5 and 20°C, and the disappearance of the starting nucleoside was quantified by use of HPLC. Both of these reactions proceeded by the pseudo-first-order kinetics, and the half lives found were 4.0 min for CF_3dUR, and 9.2 min for product I. Thus, the initial product, I, has a reactivity towards bisulfite with a potency about half that of CF_3dUR. In this treatment of product I with bisulfite, it was observed that one of the products was the disulfonate II. This observation confirmed the stepwise nature with which fluorine is released from the CF_3 group.

Low reactivity of product II towards bisulfite. When product II was treated with 0.1 M sodium bisulfite at pH 7.5 and 20°C, the disappearance of II was very slow: after 6 hr, only 1.5% of II reacted, as checked by HPLC.

DISCUSSION

The use of bisulfite to induce carbon-fluorine bond cleavage in CF_3dUR has allowed the identification of the stepwise nature in the fluorine release. A rapid substitution takes place for the first fluorine release, and the product of the type $-CF_2SO_3^-$ still retains a high reactivity to undergo substitution of the second fluorine. These results are consistent with the formation of exo-ring 5-methylene type compounds as intermediates (FIG. 3). Bisulfite is known to add readily across the 5,6-double bond of pyrimidine nucleosides to form 6-sulfonates,[11,12] a result of which is accelerated reactivities of 5-H[13] and 5-halogens.[14] We have previously reported that 5-hydroxymethyl-2'-deoxycytidine can be easily converted to its 5-methylsulfonate derivative on treatment with bisulfite.[15] In light of the present finding, it is conceivable that in that reaction, too, an intermediary formation of a 5-exo-methylene compound is involved.

In the reactions of CF_3dUR with methoxyamine,[3] as well as in its hydrolytic reactions,[2] products bearing two fluorine atoms were not isolated. It has been hypothesized that in these rections a nucleophile attacks position 6 of the pyrimidine ring, forming a 5-exo-methylene intermediate with a concomitant release of one fluoride anion (FIG. 1).[2,3,10] This step would be followed by addition of a nucleophile across the exo-

methylene linkage. Repetitions of these steps will lead to consecutive release of other fluorine atoms. Our present demonstration of the stepwise nature of the fluorine release in the reaction with bisulfite offers strong support for these previous suppositions of the reaction mechanisms.

The reactions of the 5-$CF(SO_3^-)_2$ derivative (II) with bisulfite are yet to be investigated. As described briefly regarding the nature of peak III in FIG. 2, multiple products seem to be involved. Analysis and identification of these compounds will be a subject of our future studies.

In the inhibition of thymidylate synthase by CF_3dUMP, the cysteinyl SH at the active site of the enzyme is believed to attack the 6-position of CF_3dUMP, generating a 5-exo-methlyene intermediate and thereby causing a single fluoride release.[2,3] It is conceivable that a nearby nucleophilic group of the enzyme protein would then bind to the 5-methylene linkage.[2] Although the structure of the inhibitor-enzyme complex has not been elucidated, it is known that fluoride ion is indeed released during the complex formation.[16] The bisulfite in the present study may be taken as a mimic for the nucleophiles in this enzyme molecule. Since in this model reaction bisulfite causes stepwise release of two fluorides, a titration of fluoride released during the CF_3dUMP-thymidylate-synthase complex formation would be informative in elucidating the inhibition mechanisms.

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