Bioorganic & Medicinal Chemistry Letters, Vol.1, No.5, pp. 271-276, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

Preparation and Evaluation of Optically Active 4,4-Difluorothreonine as a Potent Novel Antitumor Material

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(*Received* 11 *April* 1991)

Abstract: Both enantiomers of unnatural 4,4-difluorinated threonine were readily prepared via enzymatic optical resolution and only the corresponding (2*S*,3*S*) isomer was found to possess comparable or even stronger *in vitro* antitumor activity than the currently used 5-fluorouracil (5-FU).

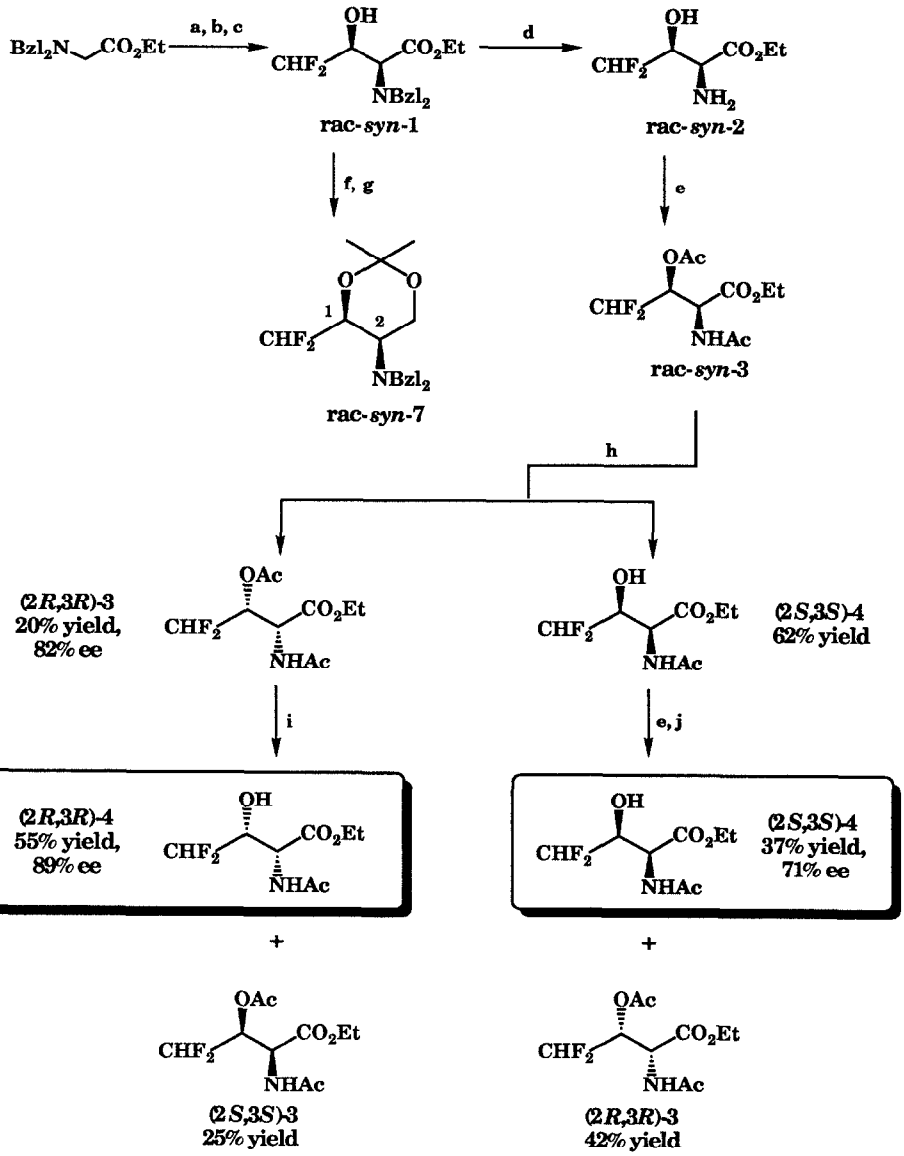
Since the pioneering work of Kollonitsch and coworkers[1] on the preparation of 3-fluoro-D-alanine-2*d*, unnatural fluorinated amino acids have drawn significant attention because they frequently have profound and unexpected results on biological activity.[2] The above amino acid, an irreversible inhibitor[3] of bacterial alanine racemase, was designed to effect enzymatic inactivation by forming a covalent bond with the active site of enzyme. Since the discovery of this artificial compound, there have been recently reported various types of fluorinated amino acids as transition state analogue inhibitors,[4] utilizing a ready hemiketal forming nature of 2,2-difluorinated carbonyl compounds.

We, on the other hand, had a special interest in fluorinated threonines (F_n-Thr[5]; n = 1, 2, or 3) not only because of their potential utility in pharmaceutical field as is reported for the other fluorinated amino acids but also their versatility as chiral building units with three easily differentiated functional groups. Previously F_1- or F_3-Thr have been synthesized in both racemic[6] and optically active forms,[7] while the corresponding F_2-derivative was, to our best knowledge, unknown even as its racemate. We would now like to report the first synthesis of optically active 4,4-difluorothreonine via the lipase-catalyzed hydrolysis[8] and the use of this difluorinated threonine as an effective antitumor agent.

The substrate for the enzymatic optical resolution was prepared as shown in Scheme I. Condensation of ethyl *N*,*N*-dibenzylglycinate with ethyl difluoroacetate, followed by reduction with $NaBH_4$ yielded racemic **1** with *syn* configuration.[9] No trace of the corresponding *anti* isomer was detected by ^1H, ^{13}C, and ^{19}F NMR spectroscopies. Deprotection of amino group and acetylation of both amino and hydroxyl moieties furnished the substrate rac-*syn*-**3** for the enzymatic resolution. Cellulase (from *Trichoderma viride*) was found to preferentially transform (2*S*,3*S*)-**3**[10] into the corresponding alcohol (2*S*,3*S*)-**4**, with the recovery of enantiomeric (2*R*,3*R*)-**3**[10]. To further enhance the optical purity, these compounds were subjected to the enzymatic process employing cellulase or lipase MY (from *Candida rugosa*[13]) independently to form the enantiomeric *N*-acetylated threoninates (Scheme I).[14] The optically active threoninates thus obtained were successfully converted into the free amino acids via usual acidic hydrolysis (7 h reflux in 1.2 N HCl; 65% yield).[15]

Both (2*S*,3*S*)- (or L-, 71% ee) or (2*R*,3*R*)- (or D-, 89% ee) amino acids prepared as above, as well as a racemic mixture with *syn* configuration, were examined for their growth inhibitory action towards tumor cell lines. Table I compares the results for *racemic F_2-Thr* with the established antimetabolite, 5-fluorouracil (5-

Scheme I



a) NaH, b) CHF_2CO_2Et, c) $NaBH_4$, d) H_2, Pd/C, e) AcCl, pyr, f) LAH, g) $Me_2C(OMe)_2/H^+$, h) Cellulase, 41 h (76% conv.), i) Lipase MY, 5 days (69% conv.), j) Cellulase, 2.5 h (47% conv.)

Table I Effect of Racemic F_2-Thr on the Growth of Various Tumor Cell *in vitro*[a]

Cell Line	IC_{50} (μg/mL)	
	rac-F_2-Thr	5-FU
L1210/c[b]	1.1	0.36
CCRF CEM[c]	1.7	0.95
PC10[d]	5.1	42.0
KB[d]	2.7	12.1

a) Each tumor cell line ($1x10^4$ cells/well) was incubated in the presence or absence of compound for 72 h. Then, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) was added for $OD^{570-700}$ measurements. IC_{50} (μg/mL) was given as the concentration at 50% inhibition of cell growth. % Inhibition = {1-($OD^{570-700}$ of sample well)/($OD^{570-700}$ of control well)}.
b) Mouse leukemia. c) Human leukemia. d) Human cancer.

FU), as a reference. A comparison of IC_{50} values clearly suggests the potential of this difluorinated amino acid as an antitumor substance, which interestingly showed more effective nature than 5-FU in inhibition of cell growth of human cancer or leukemia cells. On the other hand, the independent treatment of the mouse leukemia cell L1210/c with racemic, (2*R*,3*R*)-, and (2*S*,3*S*)-F_2-Thr revealed that only the (2*S*,3*S*) isomer, the same stereostructure as naturally occurring L-threonine (L-Thr), possessed activity (Table II). From the same Table, its antipode seemed not to inhibit the activity of (2*S*,3*S*)-F_2-Thr. Furthermore, this optically active difluorinated amino acid increased life span of mice bearing P388 leukemia with 170% of T/C value when treated with 250 mg/kg/day of (2*S*,3*S*)-F_2-Thr without any significant weight loss or gain (Table III).

To elucidate the mechanism for action, L1210/c cells were incubated for 72 h with three different con-

Table II Effect of Stereochemistry of F_2-Thr on the Growth of L1210/c Leukemic Cells *in vitro*[a]

F_2-Thr	IC_{50} (μg/mL)
racemic	3.3
(2*R*,3*R*) (89% ee)	>100
(2*S*,3*S*) (71% ee)	2.8

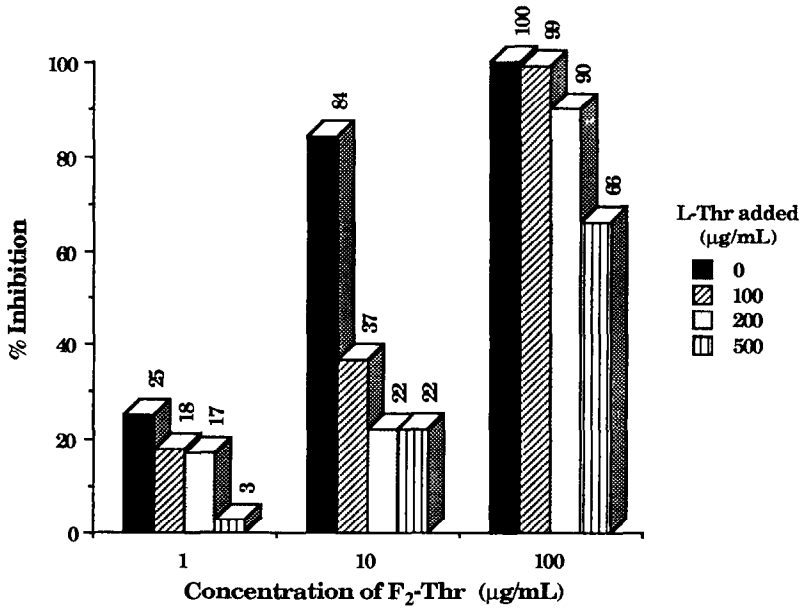
a) These experiments were conducted by the same condition as described in the footnote of Table I.

Table III Effect of (2*S*,3*S*)-F_2-Thr on the Life Span of Mice Bearing P388 Leukemia[a]

Dose (mg/kg/day)	Body Weight[b] (g) day 1	day 5	T/C (%)
20	22.0 ± 1.0	22.0 ± 1.0	114
100	21.3 ± 0.6	21.7 ± 0.6	122
250	22.7 ± 1.5	22.0 ± 1.0	170

a) Three female CDF_1 mice were implanted intraperioneally with 10^6 cells on day 0. Difluorothreonine was intraperitoneally given on days 1 to 5. T/C=(MST of the treated group/MST of the control group)x100. MST:median survival time.
b) These results are shown as the mean ± standard deviation of three mice.

Figure I Effect of F_2-Thr in the Presence or Absence of L-Thr on the Growth of Caltured L1210/c Cells



centrations of racemic difluorinated amino acid in the presence or absence of various amounts of natural L-Thr (Fig I). It is apparent that increasing the amount of racemic F_2-Thr gave a better inhibitory result. The addition of 100 μg/mL of racemic F_2-Thr led to the complete inhibition of the cell growth. On the other hand, the amount of L-Thr was found to affect this activity significantly. For example, 100 and 500 μg/mL of L-Thr, when added to the culture containing 10 and 100 μg/mL of fluorinated threonine, respectively, inhibited the control of cell growth by F_2-Thr by approximately 50% . This experiment suggested that F_2-Thr might

replace naturally occurring L-Thr in some specified system, such as in peptide synthesis. It is too early to determine the mechanistic pathway from this limited information and further study is required. But, if this is the case, difluorinated threonine mimics the behavior of natural threonine just as monofluorinated organic molecules have been shown to do previously.

In conclusion, these findings describe a new compound which possesses antitumor activity.[16] Further investigations on the mechanistic pathway as well as additional modifications of F_2-Thr are currently underway.

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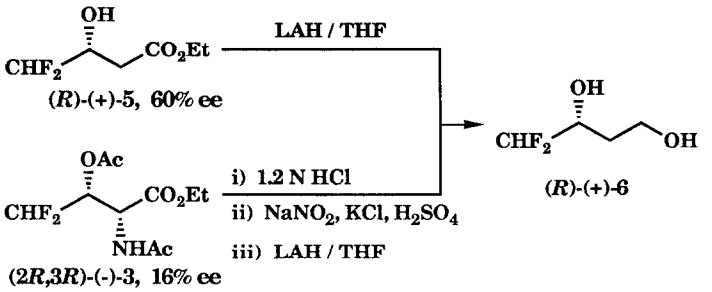
9) From the ^{1}H NMR analysis of rac-*syn*-**7** derived from rac-*syn*-**1** as is shown in Scheme I, relative stereochemistry was unambiguously established as *syn* from its coupling constant. Physical properties of rac-*syn*-**7**: R_f 0.50 (*n*-hexane:AcOEt = 5:1), mp 90.5 - 91.0 °C. ^{1}H NMR δ 1.44 and 1.45 (3 H each, s, CH_3), 2.66 (1 H, ddd, *J* = 1.08, 3.74, **4.02** Hz, C*H*N), 3.59 (2 H, d, *J* = 14.33 Hz, CH_2Ph), 3.89 (1 H, dd, *J* = 3.47, 13.17 Hz, CH_2O), 4.07 (1 H, dddd, *J* = **4.02**, 6.47, 7.59, 8.54 Hz, CHF_2CH), 4.33 (1 H, dd, *J* = 1.08, 13.17 Hz, CH_2O), 4.35 (2 H, d, *J* = 14.33 Hz, CH_2Ph), 6.20 (1 H, ddd, *J* = 6.47, 55.06, 56.07 Hz, CHF_2), 7.30 (10 H, s, Ph). ^{13}C NMR δ 18.47 and 29.24 (s, CH_3), 48.52 (dd, *J* = 2.4, 4.5 Hz, CHN), 56.21 (s, CH_2Ph), 57.76 (s, CH_2O), 72.16 (dd, *J* = 24.5, 27.6 Hz, CHOH), 99.39 (s, O-*C*-O), 114.6 (dd, *J* = 241.3, 243.4 Hz, CHF_2), 127.4, 128.8, 139.7 (s each, Ph), 169.6 (s, C=O). ^{19}F NMR (AcOEt) δ -127.2 (1 F, d, *J* = 13.5, 54.3 Hz), -127.2 (1 F, dd, *J* = 15.2, 54.3 Hz). IR (KBr) ν 2998, 1375 cm^{-1}. Anal. Calcd for $C_{21}H_{25}O_2NF_2$: C, 69.79; H, 6.97; N, 3.88. Found: C, 69.91; H, 7.08; N, 3.95.

10) Absolute stereochemistry was determined by the following chemical correlation. Thus, (2*R*,3*R*)-(-)-**3** was transformed into diol **6**, whose sign of the optical rotation ($[\alpha]_D^{20}$ +6.68° (*c* 0.61, MeOH)) was consistent with that for the same molecule obtained from configurationally known (*R*)-(+)-**5**[11] ($[\alpha]_D^{20}$ +29.10° (*c* 1.05, MeOH)). In connection with the relative configurational argument as in reference 9, stereochemistry of the compound preferentially hydrolyzed by cellulase was unambiguously determined as (2*R*,3*R*) isomer.



On the other hand, the optical purity was determined by the integration of CH_3O group after derivatization into the corresponding (*R*)-MTPA ester (δ 3.49 (q, *J* = 1.2 Hz) for (2*S*,3*S*) isomer (minor) and δ 3.58 (q, *J* = 1.3 Hz) for (2*R*,3*R*) isomer).

11) This compound was derived into 1,1-difluorononan-2-ol, whose absolute configuration has already been reported by Kobayashi and coworkers.[12] Details will be published in the near future.

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14) Physical properties of (2*R*,3*R*)-**3** (after recrystallization with AcOEt:*n*-hexane=1:1): R_f 0.33 (AcOEt:CH_2Cl_2 = 1:2), mp 134 - 135 °C. $[\alpha]_D^{26}$ -40.7° (*c* 1.28, MeOH; 82% ee) ^{1}H NMR δ 1.29 (3 H, t, *J* = 7.14 Hz, CH_3CH_2O), 2.06 (3 H, s, CH_3CO), 4.23 (2 H, q, *J* = 7.14 Hz, CH_3CH_2O), 4.26 (1 H, m, *CH*OH), 4.86 (1 H, bd, *J* = 7.24 Hz, *CH*N), 5.67 (1 H, ddd, *J* = 5.60, 55.14, 56.52 Hz, CHF_2), 5.80 (1 H, d, *J* = 7.00 Hz, O*H*), 7.10 (1 H, d, *J* = 7.24 Hz, N*H*Ac). ^{13}C NMR δ 14.11 (s, CH_3CH_2O), 22.91 (s, CH_3CO), 52.77 (dd, *J* = 2.4, 5.8 Hz, *C*HN), 62.09 (s, CH_3CH_2O), 70.85 (dd, *J* = 23.6, 27.3 Hz, *C*HOH), 115.13 (t, *J* = 244.8 Hz, CHF_2), 170.14 and 171.57 (s each, *C*=O). ^{19}F NMR (AcOEt) δ -122.0 (1 F, ddd, *J* = 7.9, 53.1, 279.1 Hz), -128.5 (1 F, ddd, *J* = 10.9, 54.0, 279.1 Hz). IR (KBr) ν 3336, 1740, 1659, 1541 cm^{-1}. Anal. Calcd for $C_8H_{13}O_4NF_2$: C, 42.67; H, 5.82; N, 6.22. Found: C, 42.80; H, 5.94; N, 6.17. (2*R*,3*R*)-**4**: R_f 0.47 (AcOEt:CH_2Cl_2 = 1:2), $[\alpha]_D^{27}$ +27.1° (*c* 0.86, MeOH, 89% ee). ^{1}H NMR δ 1.30 (3 H, t, *J* = 7.15 Hz, CH_3CH_2O), 2.10 and 2.13 (3 H each, s, CH_3CO), 4.22 (2 H, q, *J* = 7.15 Hz, CH_3CH_2O), 5.08 (1 H, ddd, *J* = 0.65, 2.10, 9.01 Hz, *CH*N), 5.53 (1 H, dddd, *J* = 2.10, 5.25, 8.58, 10.77 Hz, *CH*OH), 5.85 (1 H, ddd, *J* = 5.26, 54.15, 55.63 Hz, CHF_2), 6.27 (1 H, d, *J* = 9.01 Hz, N*H*Ac). ^{13}C NMR δ 14.01 (s, CH_3CH_2O), 20.38 and 23.04 (s each, CH_3CO), 50.84 (t, *J* = 2.6 Hz, *C*HN), 62.86 (s, CH_3CH_2O), 71.16 (dd, *J* = 22.9, 29.9 Hz, *C*HOH), 112.92 (dd, *J* = 244.8, 247.7 Hz, CHF_2), 169.34 and 170.94 (s each, *C*=O). ^{19}F NMR (AcOEt) δ -123.1 (1 F, ddd, *J* = 8.7, 53.3, 281.3 Hz), -129.4 (1 F, ddd, *J* = 11.5, 53.8, 281.3 Hz). IR (KBr) ν 3250, 2950, 1740, 1660 cm^{-1}. Anal. Calcd for $C_{10}H_{15}O_5NF_2$: C, 44.95; H, 5.66; N, 5.24. Found: C, 44.89; H, 5.60; N, 5.21.

15) Physical properties of (2*R*,3*R*)-F_2-Thr: mp 177 °C (dec). $[\alpha]_D^{24}$ +18.6° (*c* 1.24, H_2O; 89% ee). ^{1}H NMR (D_2O) δ 3.98 (1 H, d, *J* = 3.54 Hz, *CH*N), 4.46 (1 H, dddd, *J* = 2.48, 3.58, 13.12, 14.23 Hz, *CH*OH), 6.14 (1 H, dt, *J* = 2.46, 54.49 Hz, CHF_2). ^{13}C NMR (D_2O) δ 56.74 (s, *C*HN), 71.07 (t, *J* = 23.4 Hz, *C*HOH), 117.97 (t, *J* = 243.2 Hz, CHF_2), 174.73 (s, *C*=O). ^{19}F NMR (D_2O, 500 MHz) δ -130.3 (1 F, ddd, *J* = 14.1, 55.0, 288.4 Hz), -132.0 (1 F, ddd, *J* = 15.5, 53.6, 288.4 Hz). IR (KBr) ν 3282, 2900-2600, 1622 cm^{-1}. Anal. Calcd for $C_4H_7O_3NF_2$: C, 30.98; H, 4.55; N, 9.03. Found: C, 31.13; H, 4.69; N, 9.20.

16) Since chiral F_2-Thr employed here is not optically pure, some ambiguity might remain. Quantitative proof requires the optically pure materials, whose effective preparations are still underway in our laboratory.