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Potential Carcinostatics. Synthesis and Biological Properties of *d*- and *l*-β,β-Difluoroaspartic Acid and β,β-Difluoroasparagine[1]

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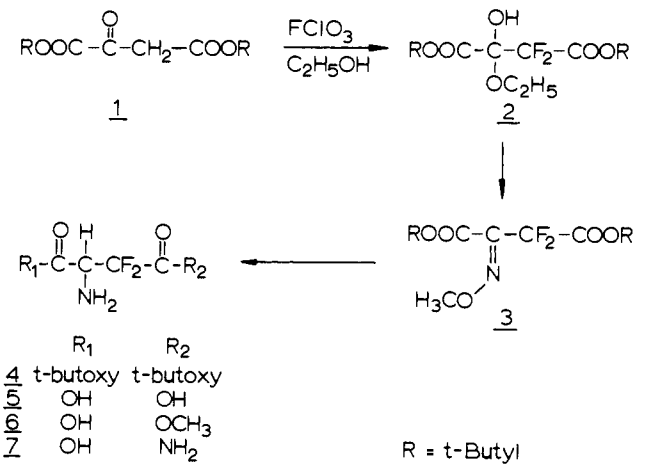
Received May 6, 1977

Di-*tert*-butyl β,β-difluorooxaloacetate, prepared by fluorination of di-*tert*-butyl oxaloacetate with perchloryl fluoride, was converted to di-*tert*-butyl β,β-difluoroaspartate via its *O*-methyl oxime, followed by reduction. The *tert*-butyl ester was hydrolyzed to give a mixture of *dl*-β,β-difluoroaspartic acid, which was resolved via its brucine salts. *dl*-Difluoroaspartic acid was converted into β,β-difluoroasparagine by monoesterification and subsequent ammonolysis. Racemic β,β-difluoroaspartic acid inhibits aspartate aminotransferase. Cell growth of 3T3-F cells was slightly inhibited by *l*-β,β-difluoroaspartic acid while the *d* enantiomer was without effect in this test system.

Interest in β,β-difluoroaspartic acid arises in view of its role as a potential inhibitor of the various aspartate-utilizing enzymes. Moreover, conversion of difluoroaspartic acid to a *reactive metabolite*, by interaction with the active site of an enzyme (e.g., via loss of HF), would allow it to act as a suicide enzyme inactivator.[2] Our special interest in β,β-difluoroaspartic acid and its suitable purine derivatives stems from the rationale that such compounds could be anticipated to behave as a reversible/irreversible inhibitor(s) of adenylosuccinate synthetase and for the adenylosuccinate lyase system.[3] The investigation forms a part of our current program on modified nucleoside analogues directed to the development of potential cytostatic agents. Interest in difluoroaspartic acid was also derived from the fact that the compound should serve as a precursor of the corresponding difluoroasparagine, which might be tested for activity in asparagine-dependent leukemias.[4] This communication describes the synthesis of optically active β,β-difluoroaspartic acids (*d*- and *l*-**5**) and racemic β,β-difluoroasparagine (**7**) and the results of preliminary biological studies of these compounds.

Chemistry. Preparation of di-*tert*-butyl β,β-difluorooxaloacetate (**2**) was accomplished via a direct fluorination of the corresponding di-*tert*-butyl oxaloacetate (**1**). Under carefully controlled conditions, using perchloryl fluoride[5] ($FClO_3$) as fluorinating reagent, **2** could be obtained. Condensation of **2** with *O*-methylhydroxylamine gave the oxime **3**.[6] Reduction of the oxime to the corresponding di-*tert*-butyl ester of difluoroaspartic acid (**4**) was carried out under a variety of conditions. The best results were obtained using aluminum amalgam in a mixture of water and diethyl ether.[7] To prepare the free racemic difluoroaspartic acid (**5**), its *tert*-butyl ester **4** was refluxed in trifluoroacetic acid. Structure **5** was confirmed by its spectroanalytical data. To obtain the enantiomers



	R_1	R_2
4	t-butoxy	t-butoxy
5	OH	OH
6	OH	OCH_3
7	OH	NH_2

of **5**, the racemic mixture was converted into the diastereomeric brucine salts. Fractional crystallization from acetone–water yielded, upon treatment of the crystalline fraction with ammonia, the ammonium salt of the enantiomer with a negative rotation. The (+) enantiomer was obtained as a crystalline product from the mother liquor. Selective esterification of *dl*-**5**, using thionyl chloride and methanol,[8] provided the monomethyl ester **6** as its hydrochloride. The IR spectrum of **6** exhibited a high carbonyl absorption at 1790 cm^{-1}, indicating that the desired carboxyl group had been esterified. Treatment of **6** with methanolic ammonia resulted in the formation of β,β-difluoroasparagine (**7**).

Biological Results. The conversion of aspartate into oxaloacetate, catalyzed by glutamate–oxaloacetate transaminase (aspartate aminotransferase E.C. 2.6.6.1), was competitively inhibited by racemic **5** (K_i = 1 mM). Cell growth of 3T3-F cells in monolayer culture was slightly inhibited by *l*-**5** (cell growth at 10 μg/mL; 75% at 100 μg/mL; 12%). In this test the *d*-**5** was inactive. With asparagine-dependent L-5178Y lymphatic leukemia, tested in vivo on CDF-1 mice, both racemic **5** and **7** were found to be inactive in nontoxic doses.

Experimental Section

All melting points are uncorrected. IR spectra were recorded on a Unicam SP-200 spectrometer and NMR spectra were run on Varian Associates Model HA-100 and XL-100 instruments, using Me_4Si as an internal standard in the case of ^1H spectra and $CFCl_3$ in the case of ^{19}F spectra. Purity of oily products was established on the basis of single spots in TLC. Microanalyses were carried out by Mr. H. Pieters of the microanalytical department of this laboratory.

Inhibition of Aspartate Aminotransferase (E.C. 2.6.6.1). The enzyme was obtained from the 100 000g supernatant of rat liver homogenate. The reaction was initiated by adding aspartate (0.5–5 mM) to 10 mM α-oxoglutarate at pH 7.5 (phosphate buffer). Concentrations of oxaloacetate were assayed by measuring the $NADH^+ \rightarrow NAD^+$ conversion at 340 nm in the presence of excess malate dehydrogenase.[9] Lineweaver–Burk plots were made at different concentrations of **5**.

Inhibition of Cell Growth. Cell growth inhibition was determined with 3T3-F cells, obtained at the Netherlands Cancer Institute as a spontaneous transformant from untransformed mouse 3T3 cells.[10] The cells were grown in Dulbecco's modification of Eagle medium supplemented with 10% newborn calf serum antibiotics. To assay the effect of **5** on cell proliferation, 5×10^5 cells were inoculated in 10-cm Petri dishes in 10 mL of regular growth medium. After 24 h, the medium was changed for a fresh one containing **5** at different concentrations. Some cultures were trypsinized to determine the number of cells per culture with the help of a Coulter counter.

Antitumoral Activity in Vivo. Intraperitoneal L-5178Y, ascitic form, was obtained by intraperitoneal inoculum of 10^6 leukemic cells suspended in 0.1 mL of Hank's solution to six CDF-1 mice. Treatment started 24 h after the inoculum of tumor cells. Animals received nine injections from day 1 to day 9. Cytostatic activity was determined by comparing median survival times with that of untreated control animals.

Di-*tert*-butyl β,β-Difluorooxaloacetate (2). To 30 g of **1**[11] (123 mmol) dissolved in 400 mL of ethanol at 0 °C, a solution of 2.9 g of sodium (126 mmol) in 40 mL of ethanol was added. After cooling to −15 °C, gaseous $FClO_3$[12] was introduced at such a rate that within 1–2 h the pH dropped to 7–8. While continuing the addition of $FClO_3$ another 2.9 g of sodium in 40 mL of ethanol was added over a period of 1.5 h. When the pH fell to 7–8, approximately 31 g of perchloryl fluoride had been taken up. The reaction mixture was, at this stage, poured into 1 L of water, whereupon the product separated. The water layer was extracted with ether; the organic layer was washed with 0.5 M NaOH and water and dried on K_2CO_3–$MgSO_4$ (1:10). In order to prevent decomposition of the *tert*-butyl ester activated by the fluorine atoms, 0.1 g of Na_2CO_3 was added to the filtrate and the solvent removed in vacuo: yield 31 g (78%) of **2** (hemiacetal) as a slightly yellow oil; IR ($CHCl_3$) 1750, 1720 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.5 [18, s, $-OC(CH_3)_3$], 1.2 (3, t, CH_3), 3.6 (2, m, OCH_2), 4.3 (1, s, OH). Drying in vacuo (P_2O_5, 0.1 mm) resulted in loss of ethanol, affording the free keto ester **2**. Anal. ($C_{12}H_{18}F_2O_5$) C, H, F.

Di-*tert*-butyl β,β-Difluorooxaloacetate *O*-Methyl Oxime (3). A solution of hemiacetal **2** (1 g, 3 mmol) and 2 g (27 mmol) of *O*-methylhydroxylamine hydrochloride in 40 mL of ethanol was kept at room temperature for 6 days. After evaporating the solvent, addition of ether to the residue precipitated the excess of the reagent. The filtrate was repeatedly shaken with portions of 2 M NaOH until the aqueous layer showed a basic pH. Drying the ether layer ($MgSO_4$) and evaporating the solvent gave oxime **3** as a colorless oil in 65% yield: IR ($CHCl_3$) 1770, 1730 (C=O), 1630 cm^{-1} (w, C=N); ^1H NMR ($CDCl_3$) δ 4.0 (3, s, OCH_3).

Di-*tert*-butyl β,β-Difluoroaspartate (4). Aluminum (100 mg) was activated with mercuric chloride.[13] A solution of 260 mg of oxime **3** in 5 mL of ether was added to the amalgam. Following addition of a few drops of water the suspension was stirred for 16 h. After filtration and evaporation the product was crystallized from pentane: yield 45%; mp 57–60 °C; IR ($CHCl_3$) 1760, 1740 (C=O), 1660 cm^{-1} ($-NH_2$); ^1H NMR ($CDCl_3$) δ 1.48, 1.52 (18, 2 s, *tert*-butyl), 4.0 (1, t, J = 12.5 Hz, CH); ^{19}F NMR ($CDCl_3$) δ −115 (d, J = 13 Hz, CF_2). Anal. ($C_{12}H_{21}F_2NO_4$) C, H, F, N.

β,β-Difluoroaspartic Acid (5). Di-*tert*-butyl ester 4 (1.88 g) was refluxed in 15 mL of trifluoroacetic acid for 1.5 h, during which time the product partly precipitated. Evaporation of the solvent and addition of ether produced 1.00 g (95%) of **5** as a white solid: mp 147 °C dec; IR (KBr) 3500–2500 (NH_3^+, COOH), 1740, 1660–1610, 1550 cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 4.69 (1, d × d, J_{H-F} = 5, J_{H-F} = 26 Hz); ^{19}F NMR (Me_2SO-d_6) δ −101, −107 (AB part of ABX system, J_{F-F} = 275, J_{H-F} = 5, J_{H-F} = 26 Hz). Anal. ($C_4H_5F_2NO_4$) C, H, F, N.

Separation of Optical Isomers of 5. To a solution of 0.5 g of racemic **5** in 20 mL of water at 100 °C was added 1.17 g of pure (−)-brucine. The clear solution was cooled to 20 °C and 40 mL of acetone was added. After 2 days at 0 °C, 0.39 g of the brucine salt crystallized out. The crystals were dissolved in 30 mL of water and after addition of a slight excess of concentrated ammonia the water layer was extracted three times with chloroform. Concentrating the water layer and several recrystallizations from water–acetone yielded 0.1 g of *l*-**5** (ammonium salt) as colorless needles: mp 131 °C dec; $[\alpha]^{20}_{578}$ −6.3° (c 0.41, water). Anal. ($C_4H_8F_2N_2O_4 \cdot H_2O$) C, H, N. The dextrorotatory enantiomer of **5** can be obtained as its brucine salt from the mother liquor. Analogous purification and recrystallizations yielded *d*-**5** (ammonium salt): mp 131 °C dec; $[\alpha]^{20}_{578}$ +4.5° (c 0.33, water). Anal. ($C_4H_8F_2N_2O_4 \cdot H_2O$) C, H, N.

Monomethyl 3-Amino-2,2-difluorosuccinate Hydrochloride (6). To 8 mL of methanol (cooled to −15 °C) was added 1.5 mL of purified thionyl chloride and the mixture stirred for 0.5 h, following which 2.5 g of amino acid **5** was added. The mixture was kept at −15 °C for 1 h and then at room temperature for 2 h. Addition of ether and filtration produced 2.3 g of **6** (hydrochloride) (72%) as a white solid (MeOH–ether): mp 169–170 °C; IR (KBr) 3200–2600, 1795, 1755 (C=O); ^1H NMR (CD_3OD) δ 3.95 (3, s, OCH_3), 5.05 (1, d × d, J_{H-F} = 4, J_{H-F} = 19 Hz); ^{19}F NMR (CD_3OD + Me_2SO-d_6) δ −105, −116 (AB part of ABX system, J_{F-F} = 265, J_{H-F} = 4, J_{H-F} = 20 Hz).

β,β-Difluoroasparagine (7). To a solution of 2.3 g of ester **6** in methanol was added, at 0 °C, 20 mL of a saturated solution of ammonia in methanol. After 1 h at room temperature, excess of ammonia and the solvent were evaporated and the residue was taken up in water. Cooling the solution yielded 1.5 g of **7** (85%) as a white solid (acetone–water): mp 180 °C dec; IR (KBr) 3350, 3200, 1710, 1650, 1590 cm^{-1}; ^{19}F NMR (D_2O) δ −105, −114 (AB part of ABX system, J_{F-F} = 265, J_{H-F} = 5, J_{H-F} = 20 Hz). Anal. ($C_4H_6F_2N_2O_3$) C, H, F, N.

Acknowledgment. The authors wish to express their thanks to Dr. G. Atassi, Free University Brussels, for the in vivo antileukemia screening, to Dr. L. Smets, Antoni van Leeuwenhoekhuis, Nederlands Kankerinstituut, Amsterdam, for in vitro cytotoxicity tests, and to Dr. A. L. Meyer, Laboratorium voor Biochemie, University of Amsterdam, B.C.P. Jansen Instituut, Amsterdam, for measuring inhibitory activities in the glutamate–oxaloacetate system.

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Bradykinin Potentiating and Sensitizing Activities of New Synthetic Analogues of Snake Venom Peptides

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The structural requirements for prolonged residual ("sensitizing") activity in bradykinin-potentiating peptides (BPP's) were investigated through a study of seven synthetic BPP's including three not previously described: [Lys^6]-BPP9a, [Gly^6]potentiator B, and [Lys^6,Gln^8]potentiator B. The quantitation of the sensitizing activities in the isolated guinea pig ileum indicated that the structural requirements for bradykinin potentiation and for sensitization were not the same. The most potent sensitizers were potentiator B and [Lys^6]-BPP9a.

Several peptides isolated from the venoms of *Bothrops jararaca*[1,2] and *Agkystrodon halys blomhoffii*,[3] when administered with bradykinin, potentiate the effects of this hormone, both in vivo and in vitro. These bradykinin-potentiating peptides are potent inhibitors of bradykininase activity,[4] but their potentiation of bradykinin effects may be partly due to a direct action on the receptors to this peptide.[5,6] Most of the natural BPP's are short-acting, and their effect on isolated smooth muscles disappears upon washing with fresh medium. One exception is the undecapeptide potentiator B (Table I), which continues to potentiate bradykinin in the isolated guinea pig ileum and rat uterus after it is removed from the medium.[3] This residual potentiation of bradykinin, termed sensitizing action, was also observed with the synthetic analogue [Gln^8]potentiator B but not with any of the other BPP's that were previously tested.[6]

The relationship between BPP structure and sensitizing activity merits further investigation because persistence of action is a desirable attribute of these compounds. In particular, it would be interesting to know whether BPP9a[7] also has sensitizing activity. This nonapeptide is one of the longer acting inhibitors of angiotensin I conversion in vivo,[8] and this activity is related to bradykinin potentiation because of the identity between angiotensin converting enzyme and bradykininase (kininase II).[8b,9]

In this paper we present the results of a determination of BPP9a's sensitizing activity and the study of three new synthetic analogues (Table I): [Gly^6]potentiator B (**3**), [Lys^6,Gln^8]potentiator B (**4**), and [Lys^6]-BPP9a (**7**). Their bradykinin-potentiating activities on the isolated guinea pig ileum preparation[10] were evaluated by the minimum concentration required for twofold potentiation, as described in detail elsewhere.[6]

In order to compare the sensitization produced by the different peptides, it was necessary to quantitate this activity, previously described only qualitatively.[3,6] The typical results obtained with [Lys^6]-BPP9a illustrate how this quantitation was achieved. Figure 1 shows that, in the presence of this peptide, bradykinin was markedly potentiated and that some potentiation remained in the five responses following removal of the BPP from the medium. No significant differences between these five responses were found for any of the BPP's tested. Although the sensitization persisted for several hours, a quantitation of this longer permanence was impaired by a slow spontaneous increase in sensitivity to bradykinin, observed in control organs left without treatment for 2 h or more. However, during 20 min taken for the five test doses, control organs showed no significant alteration of their responses to bradykinin. Accordingly, the sensitizing activity of each peptide could be estimated by the average potentiation measured in the five responses subsequent to its removal from the medium.

The effect of BPP concentration on sensitizing activity is also shown in Figure 1 for the typical case of [Lys^6]-BPP9a. Potentiating (before washout) and sensitizing (after washout) effects were concentration dependent, but the sensitizing activity reached a maximum at 0.4 μM peptide concentration. Similar observations were made with the other peptides, which differed on the maximum degree of sensitization produced. Although the minimum concentration needed for maximum sensitization also differed, this maximum could be obtained with 0.4 μM concentration of all the peptides that were studied. The bradykinin potentiating as well as the sensitizing activities of these peptides are shown on Table I. No relationship between potentiating and sensitizing activities was apparent, indicating that these two properties have different structural requirements.

The most active sensitizer was potentiator B. Replacement of either Arg^6 or Lys^8 by a neutral residue caused large losses of sensitizing potency, but the resulting analogues **2–4** still retained a significant activity (Table I). However, replacement of both basic amino acids at positions 6 and 8 by proline or glutamine residues, in potentiator C (Table I) or [Gln^8]potentiator C,[6] resulted in total loss of sensitizing activity.

We have found that BPP9a has a sensitizing activity slightly smaller than those of **2–4**. Since this nonapeptide