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^{19}F NMR Measurements – A Potential Tool for the Determination of Amino Acids in Body Fluids

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N-substituted 2,4-dinitro-6-trifluoromethylphenyl and 2-fluoro-4-nitrophenyl derivatives of several amino acids were prepared and characterized by ^{1}H, ^{13}C and ^{19}F NMR and IR spectroscopy. Fluorine chemical shift values of these compounds were found to be a good parameter for the identification of amino acids in urine and blood.

Key words: ^{19}F NMR, amino acids determination

Various amino acids, being products of the metabolic processes, are present in urine, blood and other biofluids. The normal levels of this kind of compounds encountered in body fluids of healthy organism are well known. The observation of increased concentrations of some of the amino acids or the appearance of unexpected ones may evidence the malfunction of the organism. Therefore, any simple method allowing to identify amino acids and to determine their concentrations in a straightforward way is potentially useful in disease diagnose. There are numerous reports (only few being cited here) on investigation of amino acids in urine, based on the column chromatography [1], gas-liquid chromatography [2–5] or high-performance liquid chromatography [6–10]. All these methods require a special preparation of the sample to make analyts detectable and to increase their volatility [11]. This problem may be to a large extend overcome if one uses NMR spectroscopy. The experimental procedures recommended for investigating biological samples by this method and their advantages and limitations have been discussed in review papers [12–18]. Among nuclei, ^{1}H and ^{13}C are those, which are the most often exploited in magnetic resonance investigations of biomolecules. Spectra of those nuclei bring a lot of information about the sample, but at the same time, especially ^{1}H ones, are usually complicated and not easy for interpretation. This is because the body fluids are composed of many compounds, each of them contributing to the observed spectrum and additionally each spectrum of individual component is usually complex, due to proton-proton couplings. Also ^{19}F NMR measurements are applied for the investigations discussed. They are, however, restricted to the monitoring of the bio-transformation of fluorine containing xenobiotics, *e.g.* some drugs, as there is no endogenic fluorine compound in living organisms. Actually, the lack of chemical noise is a great advantage of this kind of spectroscopy.

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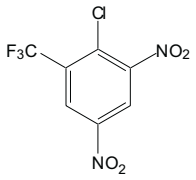
Moreover, as it is well known, the fluorine-19 is a highly sensitive nucleus and its chemical shift is very sensitive to the structural changes of the molecule. The overall ^{19}F chemical shift range is much larger than that for protons. Therefore, if one is able to label the components of investigated mixture with fluorine containing marker, ^{19}F NMR spectrum should give the information about the number, identity and quantity of mixture components. This idea was reported in regard to *N*-trifluoroacetyl derivatives of amino acids [19], but to our knowledge it was not applied for analysis of body fluids in practice. There are a few features, which fluoro compound should possess to be a good marker of amino acids. Firstly, it should efficiently, if possible quantitatively, react with amino group to form chemically stable derivatives. Secondly, fluorine grouping should be placed as closely as possible to the amino acid chain to be affected by its electronic and steric attractions. Thirdly, the markers, possessing a few magnetically equivalent fluorine atoms, if possible not coupled to protons, are preferable because of the increased sensitivity of measurements. Finally, the compound chosen as a marker, should be easily accessible. The following compounds seem to be examples, which fulfill above demands: 2-chloro-1,5-dinitro-3-trifluoromethylbenzene, **1**, 1,2-difluoro-4-nitrobenzene, **2**, 2-chloro-1-fluoro-3,5-dinitrobenzene, **3**, and 2,6-difluorobenzoil chloride, **4**.



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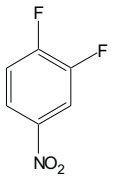

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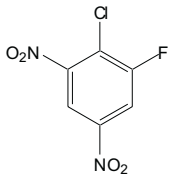

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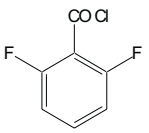

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In this paper the spectral properties of 2,4-dinitro-6-trifluoromethylphenyl derivatives of a series of amino acids are presented. Few examples of the reaction products of amino acids with 1,2-difluoro-4-nitrobenzene, **2**, less reactive equivalent of **3** [8]**,** are also reported.

RESULTS AND DISCUSSION

Three electron withdrawing groups in **1** make chlorine atom susceptible for nucleophilic, aromatic substitution with nitrogen bases. The reactions between several amino acids and **1** or **2** have been performed in alkali, ethanol-water solution. The products of general formula given in Experimental were separated and their identity proved by HRMS, ^{1}H, ^{13}C and ^{19}F NMR. Because amino acid derivatives of **1** and **2** are only slightly soluble in $CDCl_3$, acetone-d_6 has been used in the NMR measurements. The gradual replacement of *N*-hydrogen by deuterium atom has been observed even in this solvent, affecting especially ^{1}H spectra. Therefore, the sample had to be prepared just before measurement. Partial proton-deuterium exchange influences

also the fluorine chemical shift. In $H_2O - D_2O$ mixtures two signals have been usually observed with chemical shift isotope effect of the order of 0.05 ppm. Thus, it has been decided to measure the ^{19}F NMR spectra in an ordinary water solution, using the conditions being close to that encountered after treating the real biofluid sample by marker. The NMR tube (4 mm o. d.) with solution under investigation was placed in other NMR tube (5 mm o. d.) containing deuterium oxide for lock signal and 2,2,2-trifluoroethanol as a chemical shift reference. The chemical shift values measured for a series of the selected amino acid derivatives of **1** and **2** are collected in Table 1. Surprisingly, in the case of **2**, the differences between these values are small (with exception of that for Pro-**2**) and their utility for discussed purpose is limited. For derivatives of **1** those values are spread over *ca.* 4 ppm. This range is not so wide as one would wish it to be. However, even in proton coupled spectra, measured with the use of spectrometer operating at 4.7 T, all but two signals are clearly distinguishable.

Table 1. ^{19}F chemical shift values (ppm) for reference solutions of aminoacid-**1** and aminoacid-**2** compounds.

Aminoacid	Pro	Gly	Thr	Val	Leu	Ser	Gab	Glh	Ala	Phe
1 derivs.	20.64	18.27	18.19	17.86	17.48	17.39	16.87	16.87	16.70	16.41
2 derivs.	–50.99	–57.78	–57.25			–57.19	–58.07	–57.14	–57.25	–57.29

In order to check the usefulness of the method proposed in practice we applied it to analysis of urine samples. Two such samples were available. In the first one, from a child suffering from tyrosinemia, the presence of *p*-hydroxyphenyl-acetic, *p*-hydroxyphenyl-lactic and *p*-hydroxyphenyl-pyruvic acids was determined by GC-MS method (analysis of organic acids). In the second one, from a child suffering from maple syrup urine disease α-oxo-isoveleric, α-oxo-β-methylvaleric and α-oxo-isocapronic acids were found. On this basis one could expect to encounter increased concentration of tyrosine in the former sample and of leucine, isoleucine and valine in the latter one. Indeed, one and three fluorine signals have been observed, respectively, after treating investigated samples with **1**. Their chemical shift values were close within 0.4 ppm to those measured for the appropriate reference solutions. The identity of the amino acids was additionally proved by measuring the fluorine spectra after successive addition of the model derivatives and monitoring intensity of the appropriate signals. For comparison, the urine sample from a healthy child was treated with **1** in the same way as two samples described above. No fluorine signals have been found in this case. However, when the reaction with **1** was performed, using the sample of the same urine, which was concentrated about 10 times by water evaporation, several signals appeared in the fluorine spectrum. On the basis of their chemical shift values, five of them were selected as originating from derivatives of alanine, glycine, phenylalanine, serine and γ-aminobutyric acid. The above amino acid derivatives were then separated out from the obtained mixture by preparative TLC method. It was unequivocally proved by ^1H NMR spectra of obtained fractions that the above identification based on ^{19}F NMR spectra was correct.

The discussed method of labeling of amino acid with **1** was also applied to the sample of human blood. In this case two fluorine signals were noticed. On the basis of the chemical shift values, the presence of remarkable amounts of alanine and leucine in the analyzed sample was predicted. This was proved by the spectral identification of the mixture components after their separation with the use of preparative TLC method. One could indeed expect increased concentrations of the above amino acids as the blood sample was taken from the patient suffering from brain disease, *Encephalomalatia Multicystica*, (leucine) and liver cirrohosis (alanine).

The described above pilot experiments gave very promising results. Despite the measuring conditions were not standardized, the chemical shift differences between appropriate signals in reference solutions and analyzed samples never exceeded 0.4 ppm. The additional experiments, described below, showed that those differences can be easily reduced, probably below 0.1 ppm. Three basic solutions were prepared dissolving about 10 mg of each of chosen model compounds, Ala-**1**, Thr-**1** or Pro-**1**, in 1 ml of water bicarbonate solution (60 mg/ml). After fluorine spectrum of the basic solution was measured, two third of NMR tube content was replaced with the same volume of bicarbonate water solution. This procedure was repeated additionally twice. After each dilution, ^{19}F NMR spectrum was measured. Independent of the model compound used, only slight downfield shift of fluorine signal (*ca*. 0.1 ppm for 27 times diluted sample) was observed. Also the fluorine chemical shifts remained practically unchanged in the mixture of equal volumes of all three investigated basic solutions. Above measurements were done between 30–50°C. It was found for all compounds and for different concentrations that the increase of temperature by 10°C causes an upfield shift of fluorine signal by *ca*. 0.1 ppm. One cannot exclude it to be mostly the result of reference chemical shift change rather than that of solute. Anyway, from the practical point of view, the variability of the analyte concentrations and the temperature effects on ^{19}F NMR spectrum seem not to be a problem.

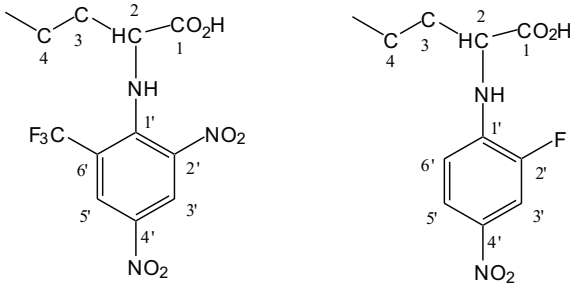
Because during workup of urine sample, ethanol has been added to homogenize the reacting mixture, the influence of this solvent on fluorine chemical shift has also been checked. For this purpose basic solutions of each of mentioned above compounds were diluted with equal volume of ethanol. Large, a few tens of ppm, upfield shift in all cases has been observed. The influence of alcohol was also proved in another experiment. Small amount of alanine was dissolved in the urine from a healthy person. The sample was treated with **1** in usual way and the fluorine spectrum of resulted solution was recorded. The chemical shift measured was 15.77 ppm. Then the sample was evaporated to half of its volume and an equal volume of water was added. After this operation, fluorine chemical shift, 16.74 ppm, was almost identical with that measured for a standard solution (see Table 1). Thus, the concentration of ethanol in the investigated solution is essential for the precision of the chemical shift measurements and has to be standardized.

The results obtained make the proposed method of amino acids determination worth further investigations. The authors are however aware of the work, which should yet be done to adopt this technique for routine, medical application. The influ-

ence of several factors (*e.g.* solution pH, interaction between different marked amino acids themselves and between them and other components of biofluids) on fluorine chemical shift has to be checked. The procedure of sample preparation may have to be modified. It would be desirable to find deuterated solvent not influencing so strongly the fluorine-19 chemical shifts as ethanol. It could serve as homogenizing agent during marking reaction and as lock reference. It would allow to resign from necessity of using tube-in-tube system during measuring of ^{19}F NMR spectra. It is also interesting to test if two or more markers can be used simultaneously for the same sample. Each of nitrogen bases should give appropriate number of fluorine signals in quite different region of the spectrum. This would certainly increase the credibility of the test. Finally, an attempt should be undertaken to apply the method discussed for quantitative amino acid determination.

EXPERIMENTAL

^{1}H and ^{13}C NMR spectra of amino acids derivatives of **1** and **2** in $^{2}H_6$-acetone solutions were recorded using a Varian Gemini 2000 spectrometer operating at 4.7 T. Residue solvent signals were used as chemical shift references for proton (δ = 2.04 ppm) and carbon spectra (δ = 29.84 ppm). The assignment of resonance signals was based on the chemical shifts, intensities and the values of H,F or ^{13}C,F coupling constants. The analysis of proton spectra has not been performed and ^{1}H,^{1}H coupling constants values were calculated as differences between the particular line positions in appropriate multiplets. Coupling constant values given for carbon spectra regard the fluorine carbon couplings. Reference solutions were prepared dissolving about 10 mg of amino acid-**1** or amino acid-**2** in 1 mL of water solution of bicarbonate (60 mg/ml). ^{19}F NMR spectra of reference solutions were measured in 4 mm NMR tubes placed in 5 mm NMR tubes containing deuterium oxide (lock reference) and a drop of 2,2,2-trifluoroethanol (chemical shift reference; δ = 0 ppm for central line of triplet). IR spectra were recorded using a Specord M80 Carl Zeiss Jena instrument. Mass spectra were obtained under electron impact.



*Preparation of amino acid derivatives of **1** and **2*** [20]: A solution of amino acid (3 mmol), **1** or **2** (3.1 mmol) and sodium bicarbonate (6.2 mmol) in the mixture of ethanol (7 mL) and water (7 mL) was refluxed for 2 h. After cooling the mixture was extracted with ether to remove unreacted marker, acidified with hydrochloric acid and thoroughly extracted with ether. Ether layer was washed with water and brine and dried with magnesium sulfate. Ether was evaporated under vacuum and residue crystallized from toluene.

Pro-**1**: M.p. 152–154°C; HRMS (EI) $C_{12}H_{10}N_3O_6F_3Na$ requires *M*, 372.0414, found 372.0400; ^{1}H NMR: 10.100 (vbs, 1H, OH), 8.780 (d, 1H, $H_{3'}$, $J_{H3',H5'}$ = 2.8 Hz), 8.670 (d, 1H, $H_{5'}$), 4.279 (dd, 1H, H_2, $J_{H2,H3A}$ = 7.3 Hz, $J_{H2,H3B}$ = 6.7 Hz), 3.710 (m, 1H, H_{5A}), 3.410 (m, 1H, H_{5B}), 2.400 (m, 1H, H_{4A}), 2.27–2.07 (m, 3H, H_{3A} + H_{3B} + H_{4B}); ^{13}C NMR: 171.90 (C_1), 148.20 ($C_{4'}$ or $C_{2'}$), 146.57 ($C_{2'}$ or $C_{4'}$), 141.77 ($C_{1'}$),

127.45 ($C_{5'}$, ^3J = 6.5 Hz), 125.64 ($C_{3'}$), 123.60 (CF_3, ^1J = 273.5 Hz), 119.59 ($C_{6'}$), 63.92 (C_2), 55.04 (C_5, ^5J = 2.8 Hz), 30.95 (C_3), 29.02 (C_4); IR (KBr, cm^{-1}): 3104, 2988, 2900, 1720, 1608, 1536, 1472, 1344.

Gly-**1**: M.p. 158°C; HRMS (EI): $C_9H_6N_3O_6F_3$ requires *M*, 309.0210, found 309.0209; ^{1}H NMR: 8.984 (d, 1H, $H_{3'}$, $J_{H3',H5'}$ = 2.9 Hz), 8.637 (d, 1H , $H_{5'}$), 8.150 (bs, 1H, NH), 4.188 (dq, 2H, H_2, $J_{H2,NH}$ = 4.8 Hz, $^5J_{H2,F}$ = 0.6 Hz); ^{13}C NMR: 170.73 (C_1, ^5J = 1.2 Hz), 146.42 ($C_{1'}$), 137.52 ($C_{4'}$ or $C_{2'}$), 136.52 ($C_{2'}$ or $C_{4'}$), 129.00 ($C_{5'}$, ^3J = 6.5 Hz), 127.49 ($C_{3'}$), 118.53 (CF3, ^1J = 272.8 Hz), 47.71 (C_2, ^5J = 3.8 Hz); IR (KBr, cm^{-1}): 3252, 3108, 2928, 2856, 1712, 1624, 1536, 1344, 1124.

Thr-**1**: M.p. 165°C; HRMS (EI): $C_{11}H_{10}N_3O_7F_3$ requires $M–H^+$, 352.0393, found 352.0381; 1H NMR: 9.021 (d, 1H, H_3, $J_{H3',H5'}$ = 2.8 Hz), 8.648 (d, 1H, $H_{5'}$), 8.279 (d, 1H, NH, $J_{NH,H2}$ = 9.6 Hz), 4.590 (qd, 1H, H_3, $J_{H3,H4}$ = 9.4 Hz, $J_{H3,H2}$ = 1.9 Hz), 4.284 (dd, 1H, H_2), 1.360 (d,1H, H_4); ^{13}C NMR: 171.48 (C_1), 148.05 ($C_{1'}$), 138.90 ($C_{4'}$ or $C_{2'}$), 137.10 ($C_{2'}$ or $C_{4'}$), 129.30 ($C_{5'}$, ^3J = 6.5 Hz), 127.41 ($C_{3'}$), 124.00 (CF3, ^1J = 272.7 Hz), 118.68 ($C_{6'}$, ^2J = 32.2 Hz), 68.14 (C_3), 65.00 (C_2, ^5J = 3.1 Hz), 21.17 (C_4); IR (KBr, cm^{-1}): 3548, 3416, 3108, 2988, 2928, 1720, 1616, 1516, 1340, 1272, 1132.

Val-**1**: M.p. 161–162°C; HRMS (EI) : $C_{12}H_{12}N_3O_6F_3$ requires *M*, 351.0678, found 351.0674; ^{1}H NMR: 9.028 (d, 1H, $H_{3'}$, $J_{H3',H5'}$ = 2.8 Hz), 8.667 (d, 1H, $H_{5'}$), 7.858 (d, 1H, NH), 4.30 (dd. 1H, H_2, $J_{H2,NH}$ = 9.0 Hz, $J_{H2,H3}$ = 3.8 Hz), 2.274 (dsept, 1H, H3), 1.074 (d, 3H, $CH_{3(A)}$, $J_{H4,H5}$ = 7.0 Hz), 0.991 (d, 3H, $C_{H3(B)}$, $J_{H5,H4}$ = 7.0 Hz); ^{13}C NMR: 171.88 (C_1), 146.27 ($C_{1'}$), 138.90 ($C_{4'}$ or $C_{2'}$), 137.50 ($C_{2'}$ or $C_{4'}$), 129.30 ($C_{5'}$, ^3J = 6.5 Hz), 127.56 ($C_{3'}$), 123.93 (CF_3, ^1J = 273.1 Hz), 119.19 ($C_{6'}$, ^2J = 32.4 Hz), 64.40 (C_2, ^5J = 3.0 Hz), 32.14 (C_3), 18.33 ($CH_{3(A)}$ or $CH_{3(B)}$), 17.82 ($CH_{3(B)}$ or $CH_{3(A)}$); IR (KBr, cm^{-1}): 3320, 3096, 2980, 1708, 1612, 1504,1344, 1268, 1132.

Leu-**1**: oil, HRMS (EI): $C_{13}H_{14}N_3O_6F_3$ requires *M*, 365.0835, found 365.0826; ^{1}H NMR: 9.025 (d, 1H, $H_{3'}$, $J_{H3',H5'}$ = 2.8 Hz), 8.665 (d, 1H, $H_{5'}$), 7.617 (d, 1H, NH, $J_{NH,H2}$ = 8.8 Hz), 4.39 (m, 1H, H_2), 1.95–1.78 (m, 3H, H_3 + H_4), 0.986 (d, 3H, $CH_{3(A)}$, $J_{CH3(A),H4}$ = 6.4 Hz), 0.928 (d, 3H, $CH_{3(B)}$, $J_{CH3(B),H4}$ = 6.0 Hz); ^{13}C NMR: 172.98 (C_1), 146.55 ($C_{1'}$), 138.80 ($C_{4'}$ or $C_{2'}$), 137.65 ($C_{2'}$ or $C_{4'}$), 129.34 (q, $C_{5'}$, ^3J = 6.5Hz), 127.47 ($C_{3'}$), 123.88 (CF3, ^1J = 271.5 Hz), 119.17 ($C_{6'}$, ^2J = 32.43 Hz), 58.35 (C_2, ^5J = 3.1 Hz), 43.12 (C_4), 25.68 (C_3), 22.70 ($CH_{3(A)}$), 22.18 ($CH_{3(B)}$); IR (KBr, cm^{-1}): 3308, 3104, 2972, 1720, 1620, 1520, 1344, 1628, 1128.

Ser-**1**: M.p. 145°C; HRMS (ESI): $C_{10}H_8N_3O_7F_3Na$ requires *M*, 362.0207, found 362.0225; ^{1}H NMR: 8.997 (d, 1H, $H_{3'}$, $J_{H3',H5'}$ = 2.8 Hz), 8.642 (d, 1H, $H_{5'}$), 8.183 (d, 1H, NH, $J_{NH,H2}$ = 9.0 Hz), 4.448 (dt, 1H, H_2), 4.171 (dd, 1H, H_{3A}, $J_{H3A,H3B}$ = –11.2 Hz, $J_{H3A,H2}$ = 2.6 Hz), 4.001 (dd, 1H, H_{3B}, $J_{H3B,H2}$ = 3.4 Hz); ^{13}C NMR: 171.30 (C_1), 146.64 ($C_{1'}$, ^3J = 1.15 Hz), 139.05 ($C_{4'}$ or $C_{2'}$), 137.27 ($C_{2'}$ or $C_{4'}$), 129.10 ($C_{5'}$, ^3J = 6.5 Hz), 127.31 ($C_{3'}$), 123.92 (CF3, ^1J = 272.8 Hz), 119.25 ($C_{6'}$, ^2J = 32.4 Hz), 62.74 (C_3), 61.19 (C_2, ^5J = 3.0 Hz); IR (KBr, cm^{-1}): 3480, 3436, 1760, 1616, 1528, 1348, 1272, 1116.

Gab-**1**: M.p. 155°C; HRMS (EI): $C_{11}H_{10}N_3O_6F_3$ requires *M*, 337.0522, found 337.05270; ^{1}H NMR: 8.890 (d, 1H, $H_{3'}$, $J_{H3',H5'}$ = 2.7 Hz), 8.570 (d, 1H, $H_{5'}$), 7.500 (bs, 1H, NH), 3.350 (dt, 2H, $J_{H4,NH}$ = 5.4 Hz, $J_{H4,H3}$ = 6.6 Hz), 2.458 (t, 2H, H_2, $J_{H2,H3}$ = 7.1 Hz), 2.089 (pent, 2H, H_2); ^{13}C NMR: 174.44 (C_1, ^6J = 1.15 Hz), 146.58 ($C_{1'}$), 136.72 ($C_{4'}$ or $C_{2'}$), 135.86 ($C_{2'}$ or $C_{4'}$), 128.45 ($C_{5'}$, ^3J = 6.5 Hz), 127.66 ($C_{3'}$), 123.90 (CF_3, ^1J = 272.7 Hz), 117.90 ($C_{6'}$, ^2J = 32.4 Hz), 47.85 (C_4, ^5J = 2.7 Hz), 31.33 (C_2), 25.66 (C_3); IR (KBr, cm^{-1}): 3452, 3316, 1712, 1616, 1520, 1246, 1128.

Glh-**1**: M.p. 161°C; LRMS (EI): $C_{12}H_{12}F_3N_4O_7$ requires *M*+H; 381.0658, found 381.0525; ^{1}H NMR: 8.962 (d, 1H, $H_{3'}$, $J_{H3',H5'}$ = 2.8 Hz), 8.628 (d, 1H, $H_{5'}$), 7.830 (d, 1H, NH, $J_{NH,H2}$ = 8.6 Hz), 7.099 (bs, 1H, NH), 6.572 (bs, 1H, NH), 4.407 (m, 1H, H_2), 2.394 (m, 2H, H_4), 2.209 (m, 2H, H_3); ^{13}C NMR: 174.90 (C_1), 172.72 (C_5), 146.03 ($C_{1'}$), 139.07 ($C_{4'}$ or $C_{2'}$), 137.40 ($C_{2'}$ or $C_{4'}$), 128.86 ($C_{5'}$, ^3J = 6.5Hz), 127.37 ($C_{3'}$), 123.79 (CF_3, ^1J = 273.1Hz), 119.40 ($C_{6'}$, ^2J = 32.4 Hz), 59.12 ($C_{6'}$, ^2J = 32.4 Hz), 59.12 (C_4), 57.70 (C_3), 18.77 (C_2); IR (KBr, cm^{-1}): 3456, 3376, 3108, 1720, 1620, 1520, 1344, 1272, 1344, 1132.

Ala-**1**: M.p. 181–182°C; HRMS (EI) : $C_{10}H_7N_3O_6F_3$ requires *M*, 322.0281, found 322.0276; ^{1}H NMR: 10.300 (vbs, 1H, OH), 8.982 (d, 1H, $H_{3'}$, $J_{H3',H5'}$ = 2.6 Hz), 8.647 (d, 1H , $H_{5'}$), 7.652 (bd, 1H, NH, JNH,H2 = 8.2 Hz), 4.350 (dq, 1H, H_2, $J_{H2,H3}$ =7.0 Hz), 1.528 (d, 3H, H_3); ^{13}C NMR: 173.52 (C_1), 144.53 ($C_{1'}$), 137.96 ($C_{4'}$ or $C_{2'}$), 136.52 ($C_{2'}$ or $C_{4'}$), 127.81 ($C_{5'}$, ^3J = 6.5 Hz), 126.25 ($C_{3'}$), 122.72 (CF_3, ^1J = 272.7 Hz), 119.21 ($C_{6'}$, ^2J = 32.0 Hz), 55.04 (C_2, ^5J = 2.7 Hz), 19.08 (C_3); IR (KBr, cm^{-1}): 3360, 3104, 2924, 2856, 1728, 1616, 1728, 1616, 1524, 1344, 1120.

Phe-**1**: M.p. 147–149°C; HRMS (ESI): $C_{16}H_{11}N_3O_6F_3$ requires *M*–H^+, 398.0600, found 398.0592; ^1H NMR: 8.937 (d, 1H, $H_{3'}$, $J_{H3',H5'}$ = 2.7 Hz), 8.603 (d, 1H , $H_{5'}$), 7.575 (d, 1H, NH, $J_{NH,H2}$ = 9.2 Hz), 7.261–7.133 (m, 5H, $H_{4,5,6,7,8}$), 4.70 (ddd, 1H, H_2, $J_{H2,H3B}$ = 6.2 Hz, $J_{H2,H3A}$ = 5.4 Hz), 3.324 (dd, 1H, H_{3A}, $J_{H3A,3B}$ = –14.2 Hz), 3.238 (dd, 1H, H_{3B}); ^{13}C NMR: 171.54 (C_1), 145.72 ($C_{1'}$), 138.62 ($C_{4'}$ or $C_{2'}$), 135.67, 129.80, 129.15, 126.94 (Ph), 137.21 ($C_{2'}$ or $C_{4'}$), 128.90 (q, $C_{5'}$, ^3J = 3.0 Hz), 127.82 ($C_{3'}$), 123.21 (CF_3, ^1J = 252.2 Hz), 118.95 ($C_{6'}$, ^2J = 28.2 Hz), 60.26 (C_2, ^5J = 2.6 Hz), 38.95 (C_3); IR (KBr, cm^{-1}): 3320, 3100, 1724, 1616, 1516, 1344, 1628, 1344, 1268, 1132.

Pro-**2**: M.p. 162–164°C; HRMS (EI): $C_{11}H_{11}N_2O_4F$ requires *M*, 254.0703, found 254.0706; ^1H NMR: 7.940 (ddd, 1H, $H_{5'}$, $J_{H5',H6'}$ = 9.2 Hz, $J_{H5',H3'}$ = 2.6 Hz, $J_{H5',F}$ = 0.4–0.6 Hz), 7.830 (ddd, 1H, $H_{3'}$, $J_{H3',F}$ = 14.8 Hz, $J_{H3',H5'}$ = 2.6Hz, $J_{H3',H6'}$ = ~0 Hz), 6.810 (t, 1H, $H_{6'}$, $J_{H6',F}$ = 9.0 Hz), 4.782 (ddd, 1H, H_2, $J_{H2,H3A}$ = 8.6 Hz, $J_{H2,H3B}$ = 5.3 Hz, $J_{H2,F}$ = 3.5 Hz), 2.100–2.600 (m, 6H, H_4–H_6); ^{13}C NMR: 173.81 (C_1, ^5J = 1.9 Hz), 149.82 ($C_{2'}$, ^1J = 241.8 Hz), 142.51 ($C_{1'}$, ^2J = 8.75 Hz), 137.44 ($C_{4'}$, ^3J = 8.3 Hz), 122.45 ($C_{5'}$, ^4J = 1.5 Hz), 115.53 ($C_{6'}$ ^3J = 5.7 Hz), 112.87 ($C_{3'}$, ^2J = 26.7 Hz), 62.71 (C_2, ^4J = 8.74 Hz), 51.34 (C_5, ^5J = 2.3 Hz), 31.74 (C_3, ^6J = 1.5 Hz), 23.64 (C_4); IR (KBr, cm^{-1}): 3432, 2988, 1724, 1660, 1608, 1524, 1316, 1208, 1076.

Gly-**2**: M.p. 155–157°C; HRMS (EI): $C_8H_7N_2O_4F$ requires *M*, 214.0399, found 214.0390; ^1H NMR: 8.000 (ddd, 1H, $H_{5'}$, $J_{H5',H6'}$ = 9 Hz, $J_{H5',H3'}$ = 2.6 Hz, $J_{H5',F}$ = 0.9 Hz), 7.900 (dd, 1H, $H_{3'}$, $J_{H3',F}$ = 11.8 Hz, $J_{H3',H5'}$ = 2.6 Hz), 6.844 (t, 1H, $H_{6'}$, $J_{H6',F}$ = 8.8 Hz), 6.150 (bs, 1H, NH), 4.20 (d, 2H, H_2, $J_{H2,NH}$ = 5.9 Hz); ^{13}C NMR: 171.16 (C_1), 149.95 ($C_{2'}$, ^1J = 241.86 Hz), 143.57 ($C_{1'}$, ^2J = 7.5 Hz), 122.80 ($C_{5'}$, ^4J = 2.31 Hz), 111.28 ($C_{3'}$, ^2J = 23.13 Hz), 111.23 ($C_{6'}$, ^3J = 4.2 Hz), 44.40 ($C_{2'}$, ^4J = 5.0 Hz); IR (KBr, cm^{-1}): 3384, 3088, 2920, 1720, 1616, 1548, 1340, 1256.

Thr-**2**: oil; HRMS (EI): $C_{10}H_{11}N_2O_5F$ requires *M*, 258.0652, found 258.0649; ^1H NMR: 8.074 (ddd, 1H, $H_{5'}$, $J_{H5',H6'}$ = 10.2 Hz, $J_{H5',H3'}$ = 2.0 Hz, $J_{H5',F}$ = 1.0 Hz), 7.968 (dd, 1H, $H_{3'}$, $J_{H3',F}$ = 9.5 Hz, $J_{H3',H5'}$ = 2.0 Hz), 6.898 (t, 1H, $H_{6'}$, $J_{H6',F}$ = 9.2 Hz), 5.780 (d, 1H, NH, $J_{NH,H2}$ = 10.6 Hz), 4.475 (qd, 1H, H_3, $J_{H3,H4}$ = 9.5 Hz, $J_{H3,H2}$ = 5.1 Hz), 4.299 (dd, 1H, H_2, $J_{H2,NH}$ = 10.6 Hz), 1.312 (d, 1H, H_4); ^{13}C NMR: 172.43 (C_1), 146.56 ($C_{2'}$, ^1J = 276.9 Hz), 122.81 ($C_{5'}$), 112.56 ($C_{3'}$, ^2J = 23.15 Hz), 111.21 ($C_{6'}$, ^3J = 4.45 Hz), 68.13 (C_3), 66.30 (C_2), 20.37 (C_4); IR (film, cm^{-1}): 3408, 3096, 1728, 1660, 1612, 1508, 1336, 1288, 1076.

Glh-**2**: M.p. 174°C; HRMS (EI): $C_{11}H_{13}N_2O_5F$ requires *M*, 286.0834, found 286.0848; ^1H NMR: 8.076 (dd, 1H, $H_{5'}$, $J_{H5',H6'}$ = 10.2 Hz, $J_{H5',H3'}$ = 1.9 Hz), 8.021 (dd, 1H, $H_{3'}$, $J_{H3',F}$ = 11.4 Hz, $J_{H3',H5'}$ = 1.9 Hz), 6.822 (t, 1H, $H_{6'}$, $J_{H6',F}$ = 10.4 Hz), 6.430 (bd, 1H, NH, $J_{NH,H2}$ = 7.0 Hz), 4.306 (m, 1H, H_2), 2.496 (m, 2H, H_4), 2.260 (m, 2H, H_3); ^{13}C NMR: 189.12 (C_1), 173.50 (C_5), 144.57 ($C_{2'}$, ^1J = 239.5 Hz), 139.78 ($C_{4'}$), 121.96 ($C_{5'}$), 112.56 ($C_{3'}$, ^2J = 23.15 Hz), 111.21 ($C_{6'}$, ^3J = 4.45 Hz), 66.31 (C4), 55.97 (C_3), 14.68 (C_2); IR (KBr, cm^{-1}): 3428, 3400, 3248, 1720, 1660, 1616, 1544, 1332.

Ser-**2**: M.p. 179°C; HRMS (EI): $C_9H_9N_2O_5F$ requires *M*, 214.0399, found 214.0390; ^1H NMR: 7.975 (dd, 1H, $H_{5'}$,$J_{H5',H6'}$ = 10 Hz, $J_{H5',H3'}$ = 3.8 Hz), 7.908 (dd, 1H, $H_{3'}$, $J_{H3',F}$ = 9.6 Hz), 6.924 (t, 1H, $H_{6'}$, $J_{H6',F}$ = 8.8 Hz), 5.951 (bd, 1H, NH, $J_{NH,H2}$ = 5.9 Hz), 4.512 (dt, 1H, H_2, $J_{H2,NH}$ = 8 Hz, $J_{H2,H3}$ = 3.18 Hz), 4.086 (dd, 1H, H_{3A}, $J_{H3A,H3B}$ = –11.1 Hz, $J_{H3A,H2}$ = 3.7 Hz), 4.134 (dd, 1H, H_{3B}, $J_{H3B,H2}$ = 3.8 Hz); ^{13}C NMR: 171.90 (C_1), 150.07 ($C_{2'}$, ^1J = 241.5 Hz), 143.06 ($C_{1'}$, ^2J = 11.1Hz), 137.75 ($C_{4'}$, ^3J = 8.0 Hz), 122.74 ($C_{5'}$, ^4J = 2.7 Hz), 111.64 ($C_{6'}$, ^3J = 2.7 Hz), 111.37 ($C_{3'}$, ^2J = 28.12 Hz), 62.60 (C_3), 58.08 (C_2); IR (KBr, cm^{-1}): 3476, 3104, 2912, 756, 1616, 1540, 1484, 1320.

Gab-**2**: M.p. 160°C; HRMS (EI): $C_{10}H_{11}N_2O_4F$ requires *M*, 242.0715, found 242.0703; ^1H NMR: 7.970 (ddd, 1H, $H_{5'}$, $J_{H5',H6'}$ = 9.2 Hz, $J_{H5',H3'}$ = 2.6 Hz, $J_{H5',F}$ = 1.2 Hz), 7.837 (dd, 1H, $H_{3'}$, $J_{H3',F'}$ = 11.8 Hz , $J_{H3',H5'}$ = 2.6 Hz), 6.917 (t, 1H, $J_{H6',F}$ = 8.8 Hz), 3.431 (t, 2H, H_4, $J_{H4,H3}$ = 7.0 Hz), 2.463 (t, 2H, H_2, $J_{H2,H3}$ = 7.0 Hz), 1.970 (pent, 2H, H_3); ^{13}C NMR: 174.63 (C_1), 152.22 ($C_{1'}$), 149.82 ($C_{2'}$, ^1J = 240.6 Hz), 136.56 ($C_{4'}$, ^3J = 8.6 Hz), 122.84 ($C_{5'}$, ^4J = 1.5 Hz), 111.09 ($C_{3'}$, ^2J = 23.15 Hz), 110.23 ($C_{6'}$, ^3J = 4.55 Hz), 42.83 (C_4, ^5J = 6.1 Hz), 31.42 (C_2), 24.54 (C_3); IR (KBr, cm^{-1}): 3400, 3100, 1712, 1620, 1536, 1488, 1308.

Ala-**2**: oil; HRMS (EI): $C_9H_9N_2O_5F$ requires *M*, 228.0546, found 228.0550; ^1H NMR: 7.974 (ddd, 1H, $H_{5'}$, $J_{H5',H6'}$ = 9.0 Hz, $J_{H5',H3'}$ = 2.7 Hz, $J_{H5',F}$ = 0.8 Hz), 7.919 (dd, 1H, $H_{3'}$, $J_{H3',F}$ = 11.8 Hz, $J_{H3',H5'}$ = 2.7 Hz), 6.831 (t, 1H, $H_{6'}$, $J_{H6',F}$ = 8.8 Hz), 6.240 (bs, 1H, NH), 4.378 (dq, 1H, H_2, $J_{H2,NH}$ = 8.0 Hz, $J_{H2,H3}$ = 4.4 Hz), 1.573 (d, 1H, H_3); ^{13}C NMR: 175.60 (C_1), 149.50 ($C_{2'}$, ^1J = 241.8 Hz), 122.81 ($C_{5'}$, ^4J = 2.4 Hz), 111.48 ($C_{3'}$, ^2J = 10.6 Hz), 111.20 ($C_{6'}$, ^3J=7.9 Hz), 51.62 (C_2, ^4J = 4.7 Hz), 18.16 (C_3); IR (film, cm^{-1}): 3404, 3100, 1728, 1660, 1612, 1508, 1328, 1288, 1076.

Phe-**2**: M.p. 173°C; HRMS (EI): $C_{15}H_{14}N_2O_4F$ requires *M*, 242.0715, found 242.0703; ^1H NMR: 7.848 (dd, 1H, $H_{5'}$, $J_{H5',H6'}$ = 4 Hz, $J_{H5',H3'}$ = 2.6 Hz), 7.796 (dd, 1H, $H_{3'}$, $J_{H3',F}$ = 11.3 Hz, $J_{H3',H5'}$ = 2.6 Hz), 7.192 (m, 1H, 5HAr), 6.683 (t, 1H, $H_{6'}$, $J_{H6',F}$ = 8.6 Hz), 4.45 (m, 1H, H_2), 3.36 (m, 1H, H_{3A}), 3.17 (m, 1H,

H_{3B}); ^{13}C NMR: 175.06 (C_1), 149.78 ($C_{2'}$, ^1J = 241.9 Hz), 143.30 ($C_{1'}$, ^2J = 11.4 Hz), 136.92 ($C_{4'}$, ^3J = 8.0 Hz), 130.40, 129.02, 127.24 (Ph), 122.90 ($C_{5'}$, ^4J = 2.5 Hz), 111.11 ($C_{3'}$, ^2J = 23.3 Hz), 111.08 ($C_{6'}$, ^3J= 4.2 Hz), IR (KBr, cm^{-1}): 3416, 3304, 1724, 1616, 1488, 1332, 1192.

Preparation of urine sample: Urine sample (7 mL) was mixed with ethanol (7 mL), **1** (0.5 mmol) and sodium bicarbonate (1 mmol). The mixture was refluxed for 2 h. Resulting solution was used for ^{19}F NMR spectrum measurement. In some cases obtained solution was concentrated to half of previous volume and equal amount of water was added. After ethanol was in this way removed one obtained much better agreement of the measured ^{19}F chemical shift values with those found for the standard solutions.

Preparation of blood sample: A blood sample (4 mL) was mixed with water (3 mL), ethanol (3 mL), **1** (0.5 mmol) and sodium bicarbonate (1 mmol). The mixture was refluxed for 3 h. The solution was separated from precipitated waxy solid by filtration and its ^{19}F NMR spectrum was recorded. The filtrate was then acidified with diluted hydrochloric acid and extracted with ether. The extract was washed with water and brine and dried with magnesium sulfate. Aminoacid-**1** compounds were separated by preparative TLC using Merc Kiesigel 60 F_{254} plates and ether-metanol mixture (9:1, v/v) as eluent. Separated compounds were washed out from silicagel with acetone. Two fractions were selected which contained Ala-**1** and Leu-**1** (R_f = 0.36 and 0.78, respectively).

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