Synthesis of *N*-[(*tert*-Butoxy)carbonyl]-3-(9,10-dihydro-9-oxoacridin-2-yl)-L-alanine, a New Fluorescent Amino Acid Derivative

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A simple synthesis of a new, highly fluorescent amino acid and of its protected derivative useful in peptide studies is described. The obtained derivative, *N*-[(*tert*-butoxy)carbonyl]-3-(9,10-dihydro-9-oxoacridin-2-yl)-L-alanine (**6**), shows intense long-wave absorption (above 360 nm) and emission (above 400 nm). The quantum yield of fluorescence of the investigated compound is very high, so it can serve as a sensitive analytical probe useful, *e.g.*, in analysis of peptide conformations.

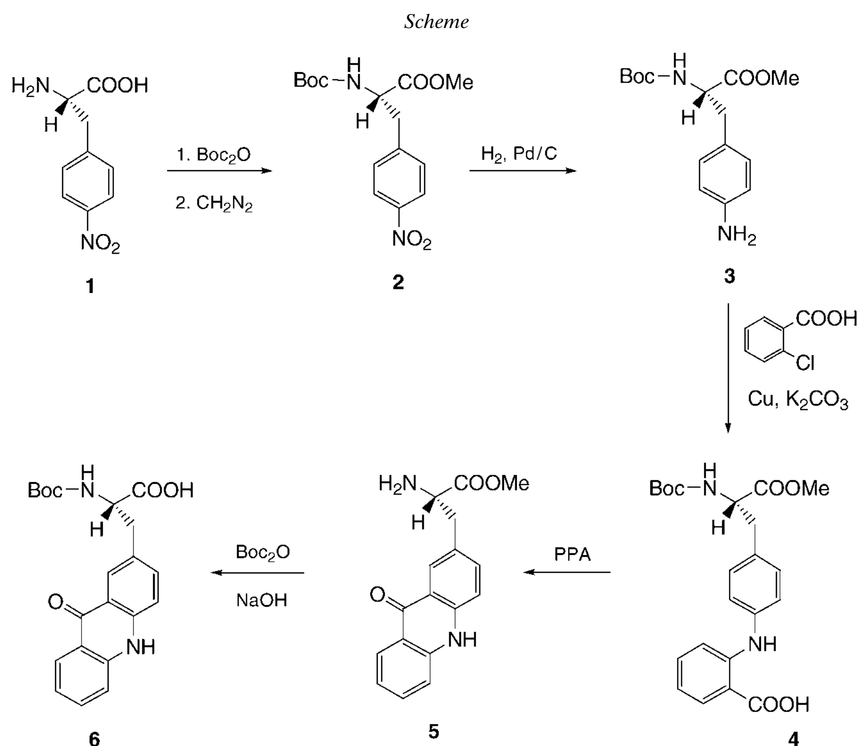
Introduction. – Labelling of peptides with designed fluorescent groups is one of the most effective and widely used analytical tool, useful for probing their structures, functions, and interactions [1–4]. Peptides can be derivatized with the fluorescent probes following their total synthesis, at the N- or C-terminus or at functional groups (NH_2, SH, or COOH) present within the sequence. However, the presence of several reactive sites can complicate this procedure. Direct incorporation of the chromophore into the peptide sequence by use of an unnatural amino acid, bearing a fluorescent function at its side chain, allows to avoid the problems mentioned. The number of available fluorescence probes is considerable [5]. Among them, special attention is paid to photostable, efficient dyes, which can be easily incorporated into a side chain of an amino acid.

Acridine and many of its derivatives (*e.g.*, 9-acridin-9(10*H*)-one) belong to the group of very effective fluorescent probes. Furthermore, the acridinone moiety is stable to light, oxidation, and heat; so it is used as a very sensitive tag in studies involving prolonged light exposure (*e.g.*, antibody catalysis [6], peptide–ligand interaction [7][8], and mass-spectrometry-based sequencing of peptides [9]). Additionally, photophysical profiles of acridinone derivatives (high quantum yield of fluorescence and long-wave shift of absorption and emission spectra [10][11]) are very beneficial from a point of view of their application as energy donors in peptide studies by means of fluorescence resonance energy transfer (FRET) [2][3][12]. These methods, based on measurements of the efficiency of the donor's electronic-excitation trapping by an appropriate acceptor, have found numerous applications in studies of macromolecules. FRET is widely used in conformational studies of biopolymers since it is capable to supply spatial data about the investigated system with a precision comparable with low-resolution X-ray-diffraction or NMR studies [12–14]. Exploring mechanisms of enzyme action and inhibition is another field of FRET applications [15–17]. Although there are many donor–acceptor pairs that can be applied in peptide studies, there is a steady need for new, rationally designed ones. In this paper, we present the synthesis of a fluorescent amino acid and its derivatives that can be used in solid-phase peptide

synthesis. On the ground of our investigation on acridine chromophores, useful in peptide studies [10][18], we decided to prepare a nonnatural amino acid bearing the acridin-9(10*H*)-one moiety, to determine its photophysical properties, and to design the appropriate donor–acceptor pairs that can be applied for conformational and enzymatic studies of peptides and proteins.

Results and Discussion. – *Synthesis. N*-[(*tert*-Butoxy)carbonyl]-3-(9,10-dihydro-9-oxoacridin-2-yl)-L-alanine (**6**; Boc-Aka-OH) was obtained from 4-nitro-L-phenylalanine (**1**) in a multi-step reaction. Thus, reduction of the 4-nitro group of the protected derivative **2** was carried out in MeOH (*Scheme*) although the solubility of the obtained **3** in MeOH is slightly lower than in THF. However, the decomposition of **3** was slower in MeOH than in THF. Due to this decomposition, the subsequent *Ullmann–Jourdan* condensation of **3** with 2-chlorobenzoic acid [10][19] was carried out under Ar to give **4** in good yield. Cyclodehydration of **4** to the acridinone derivative **5** with hot polyphosphoric acid (PPA) [20] caused concomitant removal of the Boc protecting group. To obtain an orthogonally protected amino acid suitable for peptide synthesis, we incorporated the Boc group again; under the applied basic conditions (pH 9–10, maintained by use of 1M NaOH), saponification of the ester function of **5** took place simultanously with the *N*-acylation (→**6**). Despite of a longer action of base, no racemization of the final L-alanine derivative **6** (Boc-Aka-OH) was observed.

Scheme



We determined the optical purity of **6** by reversed-phase HPLC using *Marfey*'s reagent ((2*S*)-2-[(5-fluoro-2,4-dinitrophenyl)amino]propanamide) as a differentiation agent [21]. *Marfey*'s reagent is known to form diastereoisomeric products with amino acids, which can be analyzed either by TLC or reversed-phase HPLC. A single peak corresponding to a single diastereoisomer was observed in the chromatogram of the product obtained from **6** and *Marfey*'s reagent.

Photophysical Properties. The absorption (*Fig. 1*) and fluorescence (*Fig. 2*) spectra of Boc-Aka-OH (**6**) are typical of an acridin-9(10*H*)-one substituted by a carboxyalkyl group at position 2 [10][18]. The long-wave absorption band ($\pi \rightarrow \pi^*$ transitions), interesting from a point of view of further application of the chromophore in peptide studies, is located at 360–420 nm (λ_{max} 383 and 402 nm in MeOH, λ_{max} 388 and 407 nm in H_2O). This allows to excite the acridine chromophore selectively and to avoid an interference with other natural chromophores present in an investigated peptide. Interestingly, a strong influence of the solvent on the fluorescence spectra was observed. In the case of THF, quenching of the acridinone fluorescence occurred. At this stage of the investigations, it is difficult to elucidate the mechanism of the process; we suppose that it is caused by a non-specific interaction of the chromophore with the solvent. In other solvents, the quantum yield of the fluorescence was very high.

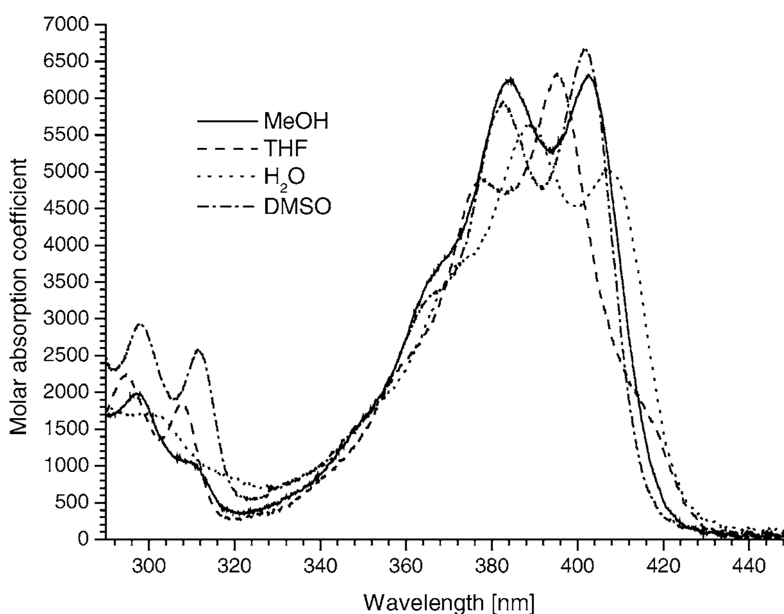


Fig. 1. *Absorption spectra of Boc-Aka-OH* (**6**) *in various solvents*

Since Boc-Aka-OH (**6**) was designed as an energy donor, we had to find an appropriate acceptor. Taking into account the rules for the donor–acceptor pairs engaged in *Förster*-type interactions [2], we chose two compounds: the well-known and widely used Dabcyl (4-[4-(dimethylamino)phenylazo]benzoyl) and a lysine derivative modified in the side chain with an anthraquinone moiety (H-Lys(AQN)-OH [22]). In

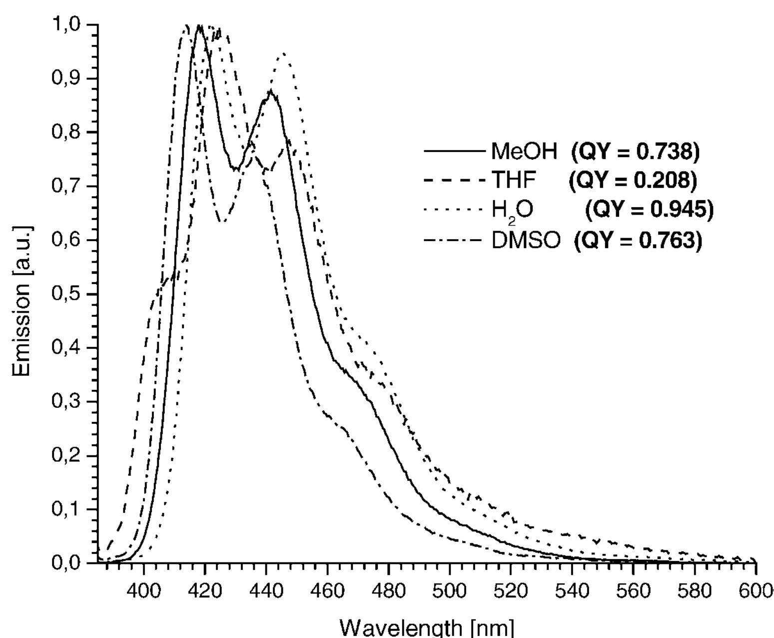


Fig. 2. *Emission spectra of Boc-Aka-OH* (**6**) *in various solvents.* QY = quantum yield.

Table. *Calculated Values of the* Förster *Distance* (R_0) *for Designed Donor–Acceptor Pairs*

Donor	Acceptor	R_0 [Å]
H-Aka-OH	Dabcyl	44.3
H-Aka-OH	H-Lys(AQN)-OH	36.4

both cases, good overlapping of the acceptor's absorption and donor's emission spectra were observed. Calculated values of the *Förster* distance are given in the *Table*.

Conclusions. We elaborated a simple and effective synthesis of a highly fluorescent amino acid in enantiomerically pure form. Excellent photophysical properties (very high quantum yield of fluorescence, photostability, long-wave absorption and emission) make this compound a very promising analytical tool, useful in many kinds of peptide investigations.

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Experimental Part

General. M.p.: uncorrected. TLC: aluminium sheets precoated with SiO_2 *60 F-254* (*Merck*); detection by UV light (λ 254 nm) or by the ninhydrin reagent. Anal. HPLC: *Kromasil* column (4.6 × 250 mm, *C-8*, 5 μm); gradient 0–100% B (B = 80% MeCN + 0.08% CF_3COOH, A = H_2O + 0.1% CF_3COOH); t_R in min. Optical rotations: *Perkin-Elmer-343* spectropolarimeter. IR Spectra: *Bruker-IFS-66* spectrometer, KBr pellets; in cm^{-1}.

NMR Spectra: CD_3OD or (D_6)DMSO solns.; *Varian-Mercury* (400-MHz) and *Tesla-BF-567A* (100 MHz) spectrometers;δ in ppm, *J* in Hz. Mass spectra: *VG-Masslab-Tri-3* spectrometer with quadrupole filter.

N-*[(*tert-*Butoxy)carbonyl]-4-nitro-*L-*phenylalanine* (Boc-Phe(4-NO_2)-OH). To the chilled (ice-bath) soln. of Phe(4-NO_2)-OH (**1**; 16.5 g, 62.6 mmol) in 1M NaOH (63 ml) and *t*-BuOH (63 ml), Boc_2O (15 g, 68.8 mmol) was added in portions during 30 min, while the pH was maintained at 8–9 by addition of 1M NaOH. The mixture was stirred for 30 min, an additional portion of *t*-BuOH (60 ml) was added, and the mixture was stirred overnight at r.t. The soln. was concentrated by evaporation of *t*-BuOH, and the alkaline residue was extracted with petroleum ether (2 × 30 ml), then acidified to pH *ca.* 2 with 1M HCl, and extracted with AcOEt (4 × 50 ml). The combined extract was washed with 1M $KHSO_4$ and brine, dried ($MgSO_4$), and evaporated: Boc-Phe(4-NO_2)-OH (15.8 g, 81.4%).Yellow oil. Crystallization from AcOEt/petroleum ether gave a crystalline solid (first crop of the crystals; 9.66 g). M.p. 108–110° ([23]: 110–112°). HPLC (reversed phase): t_R 40.97. $[\alpha]_D^{20} = +8.3$ ($c = 1.08$, MeOH) ([23]: $[\alpha]_D^{20} = +8$ ($c = 1.0$, MeOH). IR (KBr): 3362, 2985, 2930, 1716, 1685, 1520, 1350, 1166. ^{1}H-NMR (100 MHz, (D_6)DMSO): 1.37 (*s*, t_{Bu}); 3.01–3.37 (*m*, $CH_2(\beta)$); 4.05–4.22 (*m*, H–C(α)); 7.28 (br. *d*, *J* = 8.0, NH); 7.60 (*AA'BB'*, *J* = 10.0, H–C(2), H–C(6)); 8.22 (*AA'BB'*, *J* = 10.0, H–C(3), H–C(5)).

N-*[(*tert-*Butoxy)carbonyl]-4-nitro-*L-*phenylalanine Methyl Ester* (**2**). To the chilled soln. of Boc-Phe(4-NO_2)-OH (4.65 g, 15 mmol) in Et_2O (50 ml), CH_2N_2 soln. in Et_2O (prepared from *Diazogene*®, *Janssen Chemicals*, according to [24]) was added in small portions until N_2 evolution ceased. The mixture was left in an ice-bath for 30 min, warmed to r.t., and evaporated. The residue was recrystallized from Et_2O/petroleum ether: 3.07 g (63.2%) of **2**. Yellowish solid. M.p. 93–96° ([25]: 95–97°). HPLC (reversed phase): t_R 46.51. $[\alpha]_D^{20} = -8.5$ ($c = 1.03$, MeOH) ([25]: $[\alpha]_D^{20} = +30.4$ ($c = 1.055$, CH_2Cl_2). IR (KBr): 3359, 2987, 2953, 1733, 1690, 1676, 1524, 1346, 1273, 1163. ^{1}H-NMR (100 MHz, CD_3OD): 1.37 (*s*, t_{Bu}); 3.05–3.25 (*m*, $CH_2(\beta)$); 3.77 (*s*, MeO); 4.37–4.62 (*m*, H–C(α)); 7.57 (*AA'BB'*, *J* = 10.0, H–C(2), H–C(6)); 8.28 (*AA'BB'*, *J* = 10.0, H–C(3), H–C(5)).

*4-Amino-*N-*[(*tert-*butoxy)carbonyl]-*L-*phenylalanine Methyl Ester* (**3**). A soln. of **2** (4.5 g, 13.9 mmol) in MeOH (65 ml) was subjected to heterogeneous reduction with 10% Pd/C as catalyst. After completion of the reaction (2 h, TLC), the catalyst was filtered off and the filtrate evaporated: 3.86 g (94.4%) of **3**. M.p. 85–87°. $[\alpha]_D^{20} = +10.8$ ($c = 1.02$, MeOH). IR (KBr): 3398, 3375, 3235, 2984, 2951, 1742, 1691, 1517, 1170. ^{1}H-NMR (100 MHz, CD_3OD): 1.40 (*s*, ^{t}Bu); 2.75–3.05 (*m*, $CH_2(\beta)$); 3.70 (*s*, MeO); 4.13–4.45 (*m*, H–C(α)); 6.75 (*d*, *J* = 9.0, H–C(2), H–C(6)); 7.0 (*d*, *J* = 9.0, H–C(3), H–C(5)).

N-*[(*tert-*Butoxy)carbonyl]-4-[2-carboxyphenyl)amino]-*L-*phenylalanine Methyl Ester* (**4**). A mixture of **3** (3.32 g, 11.3 mmol), 2-chlorobenzoic acid (0.88 g, 5.65 mmol), K_2CO_3 (0.39 g, 2.8 mmol), freshly prepared, activated Cu powder [17] (0.225 g), and DMF (4 ml) was heated under reflux for 2 h. After cooling to r.t., the mixture was filtered, the filtrate slowly added to H_2O (30 ml), and the suspension refrigerated overnight. The oily, brownish precipitate was washed with H_2O and dried: 1.845 g (78%). The compound was dissolved in AcOEt, the soln. washed with 1M Na_2CO_3, 1M $KHSO_4$, and brine, dried ($MgSO_4$), and evaporated. The residue was purified by CC (SiO_2, AcOEt): 1.54 g (65.7%). HPLC (reversed phase): t_R 48.85. IR (KBr): 3307, 2978, 2931, 1746, 1684, 1590, 1519, 1454, 1252, 1163. ^{1}H-NMR (400 MHz, CD_3OD): 1.39 (*s*, ^{t}Bu); 2.85–2.91 (*m*, $CH_2(\beta)$); 3.70 (*s*, MeO); 4.35–4.38 (*m*, H–C(α)); 6.69–6.73 (*m*, 1 arom. H); 7.14–7.19 (*m*, 5 arom. H); 7.27–7.32 (*m*, 1 arom. H); 7.94–7.97 (*m*, 1 arom. H). ^{13}C-NMR: 27.49; 37.06; 51.43; 55.42; 113.67; 116.92; 122.04; 130.45; 132.07; 133.88; 170.65; 173.05.

*(2*S*)-2-Amino-3-(9,10-dihydro-9-oxoacridin-2-yl)propanoic Acid Methyl Ester* (**5**). Polyphosphoric acid (13 ml) was preheated to 80° and added to **4** (1.44 g, 3.74 mmol). The mixture was stirred mechanically at 80–85° for 1 h, cooled to 50°, and poured into ice-water (50 ml). The resulting precipitate was collected, washed with cold H_2O, and dried: 0.82 g (73.9%) of crude **5** as a yellow solid. Recrystallization from DMF/H_2O yielded pure **5** (0.48 g). M.p. 195–197° (dec.). HPLC (reversed phase): t_R 24.50. $[\alpha]_D^{20} = +62.9$ ($c = 1.01$, THF). IR (KBr): 3328, 2928, 2851, 1744, 1627, 1597, 1574, 1526, 1477, 1245, 1160. ^{1}H-NMR (400 MHz, (D_6)DMSO): 3.17–3.27 (*m*, $CH_2(\beta)$); 3.59 (*s*, MeO); 4.35–4.38 (*m*, H–C(α)); 7.02 (*t*, *J* = 8.0, H–C(4)); 7.53–7.55 (*m*, H–C(5), H–C(8)); 7.65–7.69 (*m*, H–C(6), H–C(7)); 8.00 (*s*, H–C(1)); 8.17–8.19 (*d*, *J* = 8.0, H–C(3)). ^{13}C-NMR: 36.24; 52.17; 53.60; 117.39; 117.80; 120.19; 120.34; 120.77; 125.86; 126.21; 133.98; 134.89; 139.96; 140.79; 170.34; 176.52. FAB-MS: 297.3 ($[M+1]^+$).

*(2*S*)-2-{[(*tert-*Butoxy)carbonyl]amino}-3-(9,10-dihydro-9-oxoacridin-2-yl)propanoic Acid* (**6**). As described above for *N*-[(*tert*-butoxy)carbonyl]-4-nitro-L-phenylalanine, from **5** (0.76 mmol; reaction time 24 h). Crystallization from THF/cyclohexane gave 0.17 g (59%) of **6**. M.p. 248–250° (dec.). HPLC: t_R 34.58. $[\alpha]_D^{20} = +114.3$ ($c = 0.50$, THF). IR (KBr): 3274, 3174, 3145, 3000, 2975, 1746, 1682, 1635, 1590, 1552, 1525, 1181. ^{1}H-NMR (400 MHz, (D_6)DMSO): 1.29 (*s*, ^{t}Bu); 2.92–2.98 (*m*, 1 H–C(β)); 3.11–3.16 (*m*, 1 H–C(β)); 4.12–4.18 (*m*, H–C(α)); 7.13 (*d*, *J* = 8.4, NH); 7.22–7.26 (*m*, H–C(4)); 7.46–7.54 (*m*, H–C(5), H–C(8)); 7.62–7.65 (*m*, H–C(6)); 7.69–7.73 (*m*, H–C(7)); 8.12 (*s*, H–C(1)); 8.22–8.24 (*d*, *J* = 8.0, H–C(3)). ^{13}C-NMR: 36.28;

37.76; 58.46; 87.99; 127.18; 127.22; 130.24; 130.39; 130.77; 135.98; 136.05; 140.75; 143.23; 144.62; 149.57; 150.74; 165.40; 183.38; 186.59.

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