Journal of Peptide Science
J. Peptide Sci. **6**: 139–144 (2000)

Synthesis of β-(1-Azulenyl)-L-Alanine as a Potential Blue-colored Fluorescent Tryptophan Analog and its Use in Peptide Synthesis

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Received 28 September 1999
Accepted 29 September 1999

Abstract: Acetyl-β-(1-azulenyl)-D,L-alanine has been synthesized in high overall yield by the malonic ester condensation procedure, and the racemate has been enzymatically resolved with acylase I from *Aspergillus melleus*. The enantiomerically pure L-amino acid is of interest as a blue-colored fluorescent tryptophan analog. The bioactivity data of a heptagastrin analog containing it suggests that the planar aromatic azulene moiety may indeed mimic the tryptophan side chain to some extent, and the spectral properties of the azulene moiety makes β-(1-azulenyl)-L-alanine of potential value as a UV and fluorescence probe in synthetic peptides, and possibly even in proteins if bioincorporation succeeds with chemically misacylated tRNAs. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: β-(1-azulenyl)-L-alanine; peptide synthesis; spectroscopic properties; synthesis; tryptophan analog

INTRODUCTION

Owing to its unique fluorescence and ultraviolet spectral properties [1,2], the non-alternating bicyclic aromatic azulene has been intensively investigated for decades [3,4]. Many derivatives of azulene have been used in a variety of medicinal applications [5], nonetheless, no synthesis of an enantiomerically pure L-amino acid containing the azulene moiety has been reported, so far.

First attempts at synthesizing this amino acid led to the racemic mixture of the acetylated derivative, but conversion to the free racemic amino acid failed [6]. More recently, the synthesis of ethyl 2-nitro-3-(1-azulenyl) propanoate was reported, which, however, could be converted only in unsatisfying low yields to the racemic amino acid by reduction of the nitro group and saponification of the ethyl ester moiety [7]. In the present study, an efficient enantioselective preparation of β-(1-azulenyl)-L-alanine (Aal) is described and its use in peptide synthesis by the Fmoc/*t*Bu chemistry is reported.

Abbreviations: Aal, β-(1-azulenyl)-L-alanine; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; ESI-MS, electron spray ionization mass spectrometry; CE, capillary electrophoresis; CD, circular dichroism; EtOH, ethanol; AcOEt, ethyl acetate; THF, tetrahydrofuran; NMP, *N*-methylpyrrolidone; HBTU, so-called *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; HATU, so-called *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; CCK, cholecystokinin; HG, human gastrin.

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MATERIALS AND METHODS

All solvents and reagents used in the synthesis were of the highest quality commercially available, and when required were further purified and dried by standard methods. TLC silica gel 60 plates were from Merck AG (Darmstadt, Germany) and compounds were visualized with the chlorine/tolidine or permanganate reagent. CD spectra were recorded on a JASCO J 715 spectro polarimeter (JASCO Corporation, Tokyo, Japan). CE was performed on a Spectra Phoresis 1000 capillary electrophoresis apparatus (TSP, Darmstadt, Germany) at 25 kV using an underivatized fused silica capillary (67 cm × 75 μm; length × ID) and 50 mM sodium borate buffer (pH 8.5); analytical HPLC was performed on Nucleosil 100/C18 columns (Macherey-Nagel, Düren, Germany) using a linear gradient of acetonitrile:2% H_3PO_4 from 5:95 to 80:20 in 13 min. Preparative HPLC was carried out on Nucleosil RP 18 (Macherey-Nagel, Düren, Germany) with a linear gradient of 0.08% TFA in acetonitrile:0.1% TFA from 10:90 to 70:30 in 90 min. ESI-MS spectra were recorded on a Perkin Elmer API 165 mass spectrometer.

Synthesis of β-(1-Azulenyl)-L-alanine (Aal)

1-Dimethylaminomethylazulene (1). A mixture of *para*-formaldehyde (0.70 g; 23.3 mmol), *N*,*N*,*N*′,*N*′-tetramethyldiaminomethane (2.71 g; 26.5 mmol) and glacial acetic (47 ml) was heated until a clear solution was obtained. The solution was cooled to 0°C and added dropwise to an ice-cold solution of azulene (5.81 g; 45.3 mmol) in 120 ml of CH_2Cl_2. After 2 h, water (240 ml) and 5% aqueous HCl (120 ml) were added and the aqueous phase was washed four times with CH_2Cl_2. The combined organic layers were reextracted with water, the aqueous phases were adjusted to pH 12 with 2 M NaOH and extracted twice with diethyl ether. The combined ether layers were washed with water, dried over Na_2SO_4 and evaporated. Yield: 7.90 g (94%) of a blue oil; TLC (diethyl ether): R_F 0.2; analytical HPLC: t_R 5.31 min.

1-Azulenylmethyltrimethylammonium iodide (2). 1-Dimethylaminomethylazulene (7.90 g; 42.6 mmol) in dry EtOH (200 ml) was treated with methyl iodide (6.66 g; 46.9 mmol). The mixture was allowed to stand at 4°C for 16 h and the precipitate was collected and dried. Yield: 13.43 g (96%) of blue crystals; analytical HPLC: t_R 5.74 min.

Diethyl acetamino-(1-azulenylmethyl)-malonate (3). Diethyl acetaminomalonate (26.71 g; 123 mmol) was added to a solution of sodium (2.73 g; 119 mmol) in dry EtOH (400 ml) under argon atmosphere. After 10 min, 1-azulenylmethyltrimethylammonium iodide (13.41 g; 41 mmol) was added, the mixture was refluxed for 2 h, diluted with water (1600 ml), adjusted to pH 7 with 5% aqueous HCl and extracted twice with diethyl ether. The combined organic layers were washed with water, dried over Na_2SO_4 and evaporated to give a blue slurry that crystallized upon standing. Yield: 13.28 g (91%) of blue needles; TLC ($CHCl_3$:AcOEt; 10:1): R_F 0.5; analytical HPLC: t_R 10.15 min; ESI-MS: $m/z = 358.4$ $[M + H]^+$; calcd for $C_{20}H_{23}NO_5$: 357.2.

Acetamino-(1-azulenylmethyl)-malonic acid (4). To a solution of diethyl acetamino-(1-azulenylmethyl)-malonate (13.03 g; 36.5 mmol) in EtOH (300 ml), 20% aqueous KOH (300 ml) was added and the mixture was heated under reflux. After 3 h, water (1300 ml) was added and the solution extracted with diethyl ether. The aqueous phase was adjusted to pH 2 with 6 M HCl, extracted twice with diethyl ether and the combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated to dryness. Yield: 8.55 g (78%) of a blue powder; analytical HPLC: t_R 6.65 min.

α-Acetamino-β-(1-azulenyl)-propanoic Acid (5). Acetamino-(1-azulenylmethyl)-malonic acid (8.25 g; 27.4 mmol) was refluxed in a mixture of THF (100 ml) and 0.2 M HCl (600 ml) under argon atmosphere for 8 h. The solution was allowed to cool to room temperature and was extracted three times with diethyl ether. The combined organic layers were washed with water, dried over Na_2SO_4 and evaporated. Yield: 5.76 g (82%) of a blue powder; analytical HPLC: t_R 7.09 min; ESI-MS: $m/z = 258.0$ $[M + H]^+$; calcd for $C_{15}H_{15}NO_3$: 257.1.

β-(1-Azulenyl)-L-alanine (H-Aal-OH, 6). To a solution of α-acetamino-β-(1-azulenyl)-propanoic acid (5.70 g; 22.15 mmol) in 0.1 M phosphate buffer (400 ml; pH 7.5) and 4 M KOH (5.54 ml; 22.15 mmol), acylase I from *Aspergillus melleus* (2.85 g) was added. After 15 h at 37°C, the enzyme was removed by filtration through an AMICON membrane (cut-off 10 kD). The filtrate was acidified to pH 2.5 with 1 M HCl and extracted twice with diethyl ether. The aqueous phase was adjusted to pH 7 with 1 M NaOH, the solvent was evaporated to a final volume of 50 ml and the solution was stored at 4°C for 15 h. Then the precipitate was collected and washed with a little

cold water and diethyl ether. Yield: 1.89 g (39.5%; i.e. 79% of the theoretically expected yield) of blue needles; analytical HPLC: t_R 4.99 min; ESI-MS: $m/z = 216.0$ $[M + H]^+$; calcd for $C_{13}H_{13}NO_2$: 215.3; CE: t_M 51.07 min.

Peptide Synthesis

Fmoc-β-(1-azulenyl)-L-alanine (Fmoc-Aal-OH, 7). To an ice-cold solution of H-Aal-OH (0.50 g; 2.32 mmol) and Na_2CO_3 (0.49 g; 4.65 mmol) in H_2O (6 ml) and dioxane (3.5 ml), Fmoc-OSu (0.78 g; 2.32 mmol) was added in small portions. After 3 h, the mixture was diluted with H_2O and extracted twice with diethyl ether. The aqueous phase was adjusted to pH 3 with 1 M HCl, extracted three times with EtOAc and the combined organic layers were dried over Na_2SO_4 and evaporated. The crude product was precipitated from chloroform with hexane. Yield: 0.83 g (81%) of blue crystals; TLC (MeCN:$CHCl_3$:AcOH; 8:1:1): R_F 0.5; analytical HPLC: t_R 11.47 min; ESI-MS: $m/z =$ 438.2 $[M + H]^+$; calcd for $C_{28}H_{23}NO_4$: 437.2.

Synthesis of H-Ala-Tyr-Gly-Aal-Nle-Asp-Phe-NH$_2$ (8). The synthesis on solid support was carried out manually at a 0.09 mmol scale using Tentagel S RAM resin with a loading of 0.26 mmol/g (Rapp Polymere, Tübingen, Germany). All couplings were performed as double couplings with a fourfold excess of Fmoc-AA-OH:HBTU:HOBt:DIEA (1:1:1:2) in NMP:CH_2Cl_2 (4:1) except for Fmoc-Aal-OH:HATU:HOAt:DIEA (1:1:1:2), which was coupled only once with a twofold excess. The Fmoc-group was cleaved with 20% piperidine in NMP:CH_2Cl_2 (4:1) in two cycles of 3 min and 12 min, respectively. Coupling efficiency was monitored by the Kaiser test. The deprotection/resin-cleavage step was performed with TFA:H_2O:TIPS (94:5:1) in 1 h, the resin was filtered off and the filtrate was diluted with water and lyophilized. The crude product was purified by preparative HPLC. Fractions containing homogeneous material as monitored by HPLC were combined and lyophilized to a fluffy powder. Yield: 20 mg (25%) of the blue peptide; analytical HPLC: t_R 8.75 min; ESI-MS: $m/z = 881.6$ $[M + H]^+$; calcd for $C_{46}H_{56}N_8O_{10}$: 880.4.

Receptor Binding Affinities

The receptor binding affinities of compound **8** were determined with plasma membrane preparations of CHO cells with overexpressed CCK-A and CCK-B/gastrin receptor using ^{125}I-BH-(Thr,Nle)-CCK-9 as tracer according to the procedure described elsewhere [8].

RESULTS AND DISCUSSION

For the synthesis of β-(1-azulenyl)-L-alanine, use was made of the well-established synthesis of amino acids via malonic ester condensation (Figure 1). To introduce a leaving group for the nucleophilic displacement reaction, azulene was aminomethylated with *N,N,N′,N′*-tetramethyldiaminomethane, *para*-formaldehyde and acetic acid and subsequently converted to the corresponding quarternary ammonium salt **2** with methyl iodide [9]. Reaction of **2** with diethyl acetaminomalonate sodium salt gave the azulenylmethyl derivative **3**, which upon saponification of the ethyl ester moieties and decarboxylation of the corresponding α-dicarboxylic acid **4**, yielded the racemic acetyl amino acid **5**.

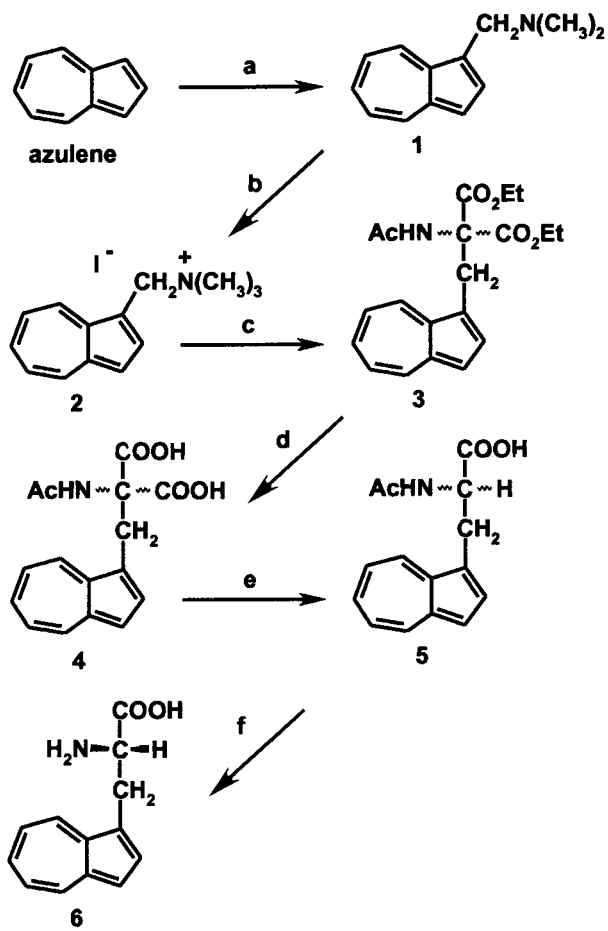

Figure 1 Synthesis of β-(1-azulenyl)-L-alanine (**6**): (a) $(CH_3)_2NCH_2N(CH_3)_2$, HCHO, AcOH, CH_2Cl_2 (94%); (b) MeI, EtOH (96%); (c) $AcNH(CO_2Et)_2$, NaOEt, EtOH, 2 h reflux under an argon atmosphere (91%); (d) 10% KOH in 50% EtOH, 3 h reflux (78%); (e) 0.2N HCl in 15% THF, 8 h reflux under an argon atmosphere (82%); (f) see [10].

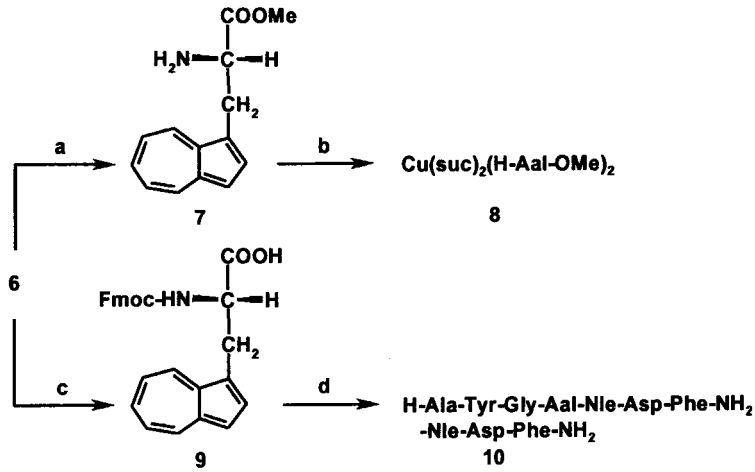

Figure 2 Synthesis of Cu(succinimidato)$_2$(H-Aal-OMe)$_2$ (**8**) and [Aal^{14}, Nle^{15}]-HG-(11–17) (**10**): (a) CH_2N_2/Et_2O, MeOH, H_2O (71%); (b) Cu(suc)$_2$(isopropylamine)$_2$, CH_2Cl_2 (*in situ*); (c) Fmoc-OSu, Na_2CO_3, dioxane/H_2O (81%); (d)i. Fmoc/*t*Bu solid phase chemistry, ii. TFA:H_2O:TIPS (94:5:1).

Enantioselective deacetylation of **5** with acylase I from *Aspergillus melleus* [10] produced β-(1-azulenyl)-L-alanine (**6**). To determine the absolute configuration of the amino acid, the related methyl ester H-Aal-OMe was prepared by reaction with diazomethane in methanol and then complexed with Cu(succinimidato)$_2$(isopropyl amine)$_2$ in dichloromethane to produce Cu(succinimidato)$_2$(H-Aal-OMe)$_2$ (**8**) (Figure 2). The CD spectrum of **8** with its minimum at 600 nm and maximum at 740 nm (Figure 3), when compared with the Cu(succinimidato) complexes of H-D-Trp-OMe and H-L-Trp-OMe [11], clearly confirmed the absolute *S*-configuration of the amino acid **6** as expected from the enzymatic resolution of the racemate **5**.

The fluorescence properties of β-(1-azulenyl)-L-alanine are reported in Figure 4. The excitation spectrum shows two major absorption maxima at 276 nm and at 339 nm, respectively, which can be used for excitation of the non-alternating aromatic ring system to yield emission at 381 nm. Compared with tryptophan with its excitation maximum at 280 nm and emission maximum at 348 nm (in aqueous buffer, neutral pH) [12], β-(1-azulenyl)-L-alanine can be excited selectively at 339 nm to yield the strongly red-shifted emission at 381 nm. Although the relative fluorescence intensity of β-(1-azulenyl)-L-alanine, when excited at 339 nm, is lower than that of tryptophan (by a factor of about 3), this amino acid could represent an interesting fluorescence probe in peptides and proteins as a potential blue-colored tryptophan analog.

By applying the selective pressure incorporation (SPI) method for expression of protein mutants *in vivo* in auxotrophic host cells, the authors recently succeeded in bioincorporation of various non-natural amino acids [13–16], including halogenated tryptophan derivatives [17]. However, attempts to incorporate β-(1-azulenyl)-L-alanine as a potential tryptophan analog into annexin V as a model protein for the production of a blue-colored mutant in Trp-auxotrophic *E. coli* failed. This most probably results from the expanded ring system or from the

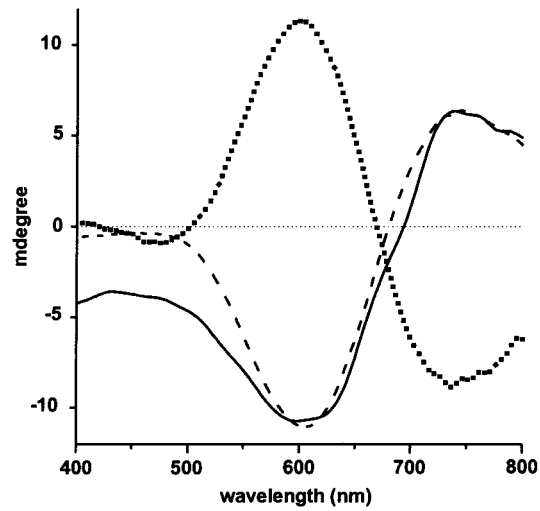

Figure 3 CD spectrum of Cu(succinimidato)$_2$(H-Aal-OMe)$_2$ (**8**) (solid line) and of the corresponding complexes of H-D-Trp-OMe (dotted line) and H-L-Trp-OMe (dashed line), recorded in CH_2Cl_2.

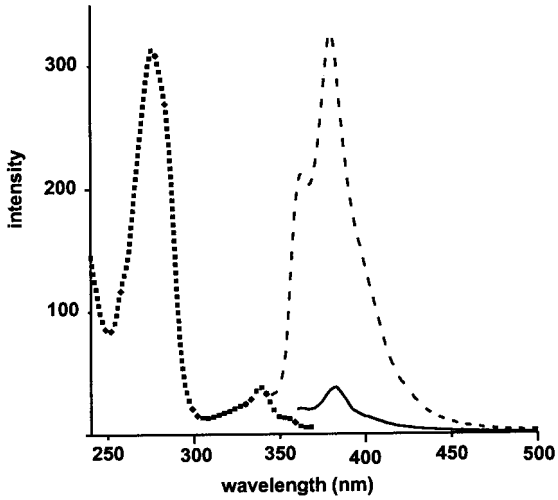

Figure 4 Fluorescence spectra of β-(1-azulenyl)-L-alanine (**6**); $c = 10^{-6}$ M in 0.1 M K_3PO_4 buffer, pH 7.5; dotted line: excitation ($\lambda_{max} = 276$ nm and 339 nm); dashed line: emission ($\lambda_{ex} = 276$ nm, $\lambda_{max} = 381$ nm); solid line: emission ($\lambda_{ex} = 339$ nm, $\lambda_{max} = 381$ nm).

lack of the indole NH-function, which prevents recognition by the tryptophanyl-tRNA synthetase. The peptide hormon gastrin contains in its bioactive portion a tryptophan residue that was proven to be essential for recognition by the receptor and thus for eliciting the hormonal response [18]. To further analyse whether β-(1-azulenyl)-L-alanine represents a potential candidate analog of Trp, the heptagastrin analog [Aal^{14}, Nle^{15}]-HG-(11–17) was synthesized by replacing the Trp residue with Aal. This analog was prepared on solid support by standard procedures of Fmoc/*t*Bu chemistry and correspondingly N^{α}-Fmoc-Aal-OH (**9**) was used as intermediate. The Fmoc derivative (**9**) was found to be stable to the conditions used, including the final acid resin-cleavage and deprotection step. The resulting blue heptagastrin (**10**) showed reduced binding affinities to the CCK-A and CCK-B receptors, overexpressed in CHO cells ($IC_{50} = 0.64$ μM for CCK-A and 0.18 μM for CCK-B receptor versus $IC_{50} = 1.6$ nM for CCK-B receptor of the related Pyr^{10}, Nle^{15}-HG-(10–17) [8]), but it retained full efficacy, although at lower potency, in inositoltriphosphate production in intact CHO cell assays ($EC_{50} = 34$ nM versus EC = 0.12 nM for Pyr^{10}, Nle^{15}-HG-(10–17) [8]).

The results obtained with the synthetic gastrin analog confirm that the planar aromatic azulene moiety can mimic the aromatic side chain of tryptophan, at least to some extent, as previously speculated by Hudson *et al.* [19]. This azulene-containing amino acid might represent an interesting spectroscopic probe in peptides when incorporated synthetically, and possibly in proteins by the use of chemically misacylated tRNAs [20].

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