Pergamon

Tetrahedron 57 (2001) 4589–4598

TETRAHEDRON

Synthesis and characterization of 4-ethoxymethylene-2-[1]-naphthyl-5(4*H*)-oxazolone and its fluorescent amino acid derivatives☆

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Received 22 October 2000; revised 23 February 2001; accepted 15 March 2001

Abstract—4-Ethoxymethylene-2-[1]-naphthyl-5(4*H*)-oxazolone **1b** was synthesized from 1-naphthoyl-glycine and triethyl orthoformate as a fluorescent analogue of the hapten 4-ethoxymethylene-2-phenyl-5(4*H*)-oxazolone. A simple and general method has been devised to prepare conjugates of type **6** between **1b** and amines and amino acids by direct reaction. Compounds **6** are stable in solid form and in aqueous solution at 20°C, but are a mixture of two isomers. ^{1}H- and ^{13}C-NMR experiments on model compounds have identified the more stable isomer. Comparative analysis of fluorescence properties of **1b** and of amino acid derivatives has identified the spectral properties of two chromophores. An intense and pH-sensitive emission band centered at λ=460 nm (λ_{ex}=360 nm) in aqueous solution has been discovered. This feature might be useful for monitoring cellular compartments of different pH.

1. Introduction

In basic immunology 4-ethoxymethylene-2-phenyl-5(4*H*)-oxazolone **1a** has received a great deal of interest.[1,2] Administration of **1a** induces a strong T-cell response.[3,4] This compound has also been used as a hapten for the analysis of antibody recognition.[5,6] It reacts with the amino groups of proteins as well as with primary amines (Fig. 1). The aminolysis of **1a** occurs by unexpected displacement of the ethoxy group by the amine with elimination of ethyl alcohol to give a 4-(substituted-methylene)-2-phenyl-5(4*H*)-oxazolone type **2a** product.[7–9] Compound **1a** was first synthesized during war-time efforts to produce penicillin.[10] The interesting chemical reactivity of this compound and its application in various fields of biological research prompted us to synthesize a new

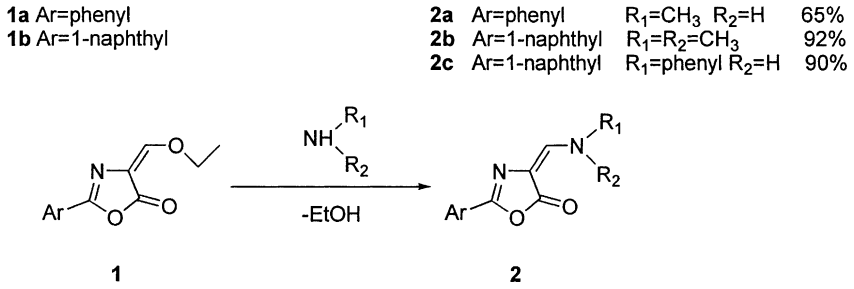

Figure 1. The reaction of 4-ethoxymethylene-2-aryl-5(4H)-oxazolone with amines.

☆ A preliminary account of this work was read at the 4th International Symposium on Amino Acids, Vienna, Austria, 1995.

Keywords: pH sensitive fluorescent chromophore; 4-ethoxymethylene-2-aryl-5(4*H*)-oxazolones; amino acid conjugate of 4-ethoxymethylene-2-[1]-naphthyl-5(4*H*)-oxazolones; alkylation of amino acid.

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0040–4020/01/$ - see front matter
PII: S0040-4020(01)00332-5

ethoxymethylene-aryl-oxazolone derivative possessing fluorescent properties. For this we have incorporated the 1-naphthyl group as the aryl substituent of the oxazolone ring at position 4 to produce **1b**. In addition we have aimed at the synthesis of type **2b** amino acid conjugates of the new fluorescent compound **1b** by utilizing the pronounced reactivity of the ethoxymethylene moiety towards the amino group.

In this communication we describe the synthesis of 4-ethoxymethylene-2-naphthyl-5(4*H*)-oxazolone, **1b**. The preparation and characterization of several amine and amino acid derivatives produced by *N*-alkylation are also reported. The detailed analysis of the spectroscopic properties of **1b** and its type **6** amino acid conjugates suggest that these compounds are fluorescent, possessing beneficial spectral characteristics. Based on these observations, we propose the application of **1b** as a new fluorophore for labelling proteins and peptides and also the incorporation of type **6** amino acid conjugates into peptides by chemical synthesis.

2. Results and discussion

2.1. Synthesis

4-Ethoxymethylene-2-[1]-naphthyl-5(4*H*)-oxazolone, like 4-ethoxymethylene-2-phenyl-5(4*H*)-oxazolone[7,10] was obtained by heating the corresponding acyl-glycine (**3**) with acetic anhydride and triethyl orthoformate (Fig. 2). For the preparation of **1b**, 1-naphthoyl-glycine was used as starting material. 1-Naphthoyl-glycine[11] was synthesized by a previously described method using different reaction conditions (e.g. benzene has been replaced by dioxane) and a more detailed description is therefore provided. The resulting compound possessed a higher and narrower melting point range when compared to the published data.

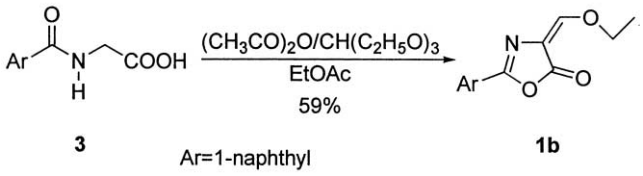

Figure 2. The synthesis of 4-ethoxymethylene-2-[1]-naphthyl-5(4H)-oxazolone **1b**.

The reaction of 1-naphthoyl-glycine with acetic anhydride and TEOF was performed in EtOAc under reflux conditions. The crystalline product **1b** was obtained after 3 h with satisfactory isolated yield (59%) and was found to be stable at +4°C for a longer period of time (>24 months). Similarly, it does not decompose in cooled non-aqueous solutions (>1 month).

Aminolysis of **1b** by amino acids yielded type **6** derivatives (Fig. 3). In order to avoid oxazolone ring opening by hydrolysis, non-aqueous reaction conditions have been used.[10] The reaction was carried out in a heterogeneous phase; amino acid (**5**) was suspended in methanol and

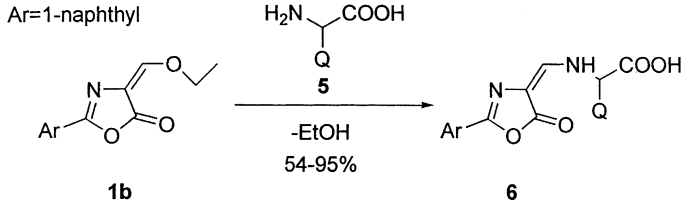

Figure 3. The synthesis of amino acid derivatives type **6** of 4-ethoxymethylene-2-[1]-naphthyl-5(4H)-oxazolone **1b**.

Table 1. Characteristics of amino acid derivatives of **1b**

Compound		Formula/molar mass	Yield (%)	Mp (°C)	Elemental analysis (calcd/found)			R_f (TLC)[a]		R_t (HPLC)[b]	
Code	Amino acid				C	H	N	System A	System B	Isomer 1	Isomer 2
6a	Gly	$C_{16}H_{12}N_2O_4$/296.28	81	207 (dec.)	64.86/64.89	4.08/4.08	9.46/9.41	0.24	0.79	**8.3**[c]	11.1**
6b	L-Ala	$C_{17}H_{14}N_2O_4$/310.30	87	148	65.80/65.78	4.55/4.59	9.03/9.10	0.34	0.85	**11.1**	15.4**
6c	D-Ala	$C_{17}H_{14}N_2O_4$/310.30	85	149	65.80/65.83	4.55/4.60	9.03/9.13	0.34	0.85	**11.9**	16.3**
6d	L-Leu×DCHA	$C_{32}H_{43}N_3O_4$/533.70	95	129	72.01/72.15	8.12/8.23	7.87/7.73	0.51	0.86	**23.5**	27.4**
6e	D-Leu×DCHA	$C_{32}H_{43}N_3O_4$/533.70	54	130	72.01/72.19	8.12/8.01	7.87/7.69	0.51	0.86	**23.3**	27.2**
6f	L-Ile	$C_{20}H_{20}N_2O_4$/352.38	87	81	68.17/68.10	5.72/5.62	7.95/8.09	0.62	0.90	**23.2**	27.6**
6g	L-Pro	$C_{19}H_{16}N_2O_4$/336.34	78	103	67.85/67.81	4.79/4.71	8.33/8.27	0.24	0.74	13.5[d]	
6h	L-Phe	$C_{23}H_{18}N_2O_4$/386.40	87	186	71.49/71.55	4.70/4.70	7.25/7.20	0.46	0.88	**24.7**	27.0**
6i	D-Phe	$C_{23}H_{18}N_2O_4$/386.40	81	186	71.49/71.48	4.70/4.64	7.25/7.18	0.46	0.88	**24.0**	27.0**
6j	L-His	$C_{20}H_{16}N_4O_4$/376.37	83	214	63.82/63.60	4.28/4.16	14.89/14.65	0.00	0.52	**29.9**	30.7*
6k	L-Trp	$C_{25}H_{19}N_3O_4$/425.44	67	109	70.58/70.45	4.50/4.41	9.88/9.73	0.34	0.84	**40.8**	42.4***
6l	α-Boc-L-Lys	$C_{25}H_{29}N_3O_6$/467.51	85	157	64.23/64.31	6.25/6.28	8.99/8.98	0.49	0.91	**37.9**	39.5****

[a] TLC, system A: pyridine/acetic acid/H_2O/EtOAc (20:6:11:333, v/v/v/v); system B: *n*-butanol/acetic acid/H_2O (4:1:1, v/v/v).
[b] RP-HPLC, column: C_{18} ODS, 300 Å, 10 μm (150×3.9 mm I.D.); eluent A: 0.1% TFA/H_2O, B: 0.1% TFA/MeCN–H_2O (80:20, v/v); v=1 mL min^{-1}; gradients: *5–60% B in 30 min; **40–80% in 30 min; ***5–60% in 45 min; or ****10–100% in 40 min.
[c] R_t value of the more stable isomer peak is printed in bold.
[d] Only a single peak is detectable.

solid **1b** was added. In this way amino acid conjugates could be obtained in fairly good yields (Table 1). The products were readily purified. Minor variations of the work-up procedure were required in a few cases. The purity of the compounds was checked by TLC using two solvent systems and by analytical RP-HPLC. Most of the compounds had sharp melting points (Table 1). Apart from isomerization (see below) no decomposition of compounds **6** was detected in solid form at −20°C.

2.2. Analysis of the isomers

The RP-HPLC analysis of compounds **6** showed two components in accordance with the finding that compounds of type **2**[7,12] and also 2-phenyl-4-(thienylmethylene)-5(4*H*)-oxazolones[13] can exist as a mixture of geometric isomers. Although baseline separation of the two isomers has been achieved (data not shown) for compounds **6**, except for the Pro derivative, HPLC attempts to isolate the components by preparative RP-HPLC under conditions applied at RT have failed. This might be due to fast isomerization in the acidic aqueous solvents used as eluents.[12] During storage in organic solution the isomeric composition of compounds of type **6** may change over time. For example in the case of **6i** the relative intensity of the two peaks in the HPLC chromatogram measured at λ=214 nm changed from 0.88 to 2.40 in 1 mg mL^{-1} acetone solution at RT during 20 h. However, no evidence was found for the isomerization of deep-frozen solid samples.

In order to assign the isomer components of the RP-HPLC chromatograms, amine derivatives of **1b** with simplified structures **2a**, **2b**, **2c** were synthesized. Compound **1b** was reacted with methylamine, *N*,*N*-dimethylamine or aniline in CH_2Cl_2. Products were obtained with a narrow melting point range and acceptable yield (65–92%). In the HPLC chromatogram of **2a**, two peaks could be detected with R_t=13.43 and 19.5 min. Similarly, compound **2c** also showed two components with R_t=12.85 and 18.3 min. In both cases the first peak corresponded to the more stable isomer. The *N*,*N*-dimethylamine derivative, **2b** displayed only a single peak under identical conditions. All three compounds are insoluble in water, but soluble in $CHCl_3$. These model compounds together with the L-Ala derivative of **1b** (**6b**) were dissolved in $CDCl_3$ and for the structural assignments ^{1}H- and ^{13}C-NMR spectra were measured.

We have constructed possible structures for the analysis of molecules with an *N*-monosubstituted amino group **2a**, **2c**, **6b** (Fig. 4). On this basis we find that two tautomers could be considered (types I and II). Owing to the presence of the double bond between C-4 and C-6, tautomer type I shows geometric isomerism (isomers I_1 and I_2). Assuming a C(6)=N double bond due to hindered rotation, two geometric isomers of type II could be drawn (II_1 and II_2). In addition, due to the free rotation about the single bond between C-4 and C-6, both isomers of tautomer type II could adopt two rotameric forms (II_1 and II_1' for the *trans*- and, II_2 and II_2' for the *cis*-isomer). Among the structures described in Fig. 4, I_1 and II_1 can be stabilized by chelation.

NMR data are summarized in Tables 2 and 3. Assignments of ^{1}H- and ^{13}C-NMR spectra for *N*-methyl-substituted compound **2a** indicate the presence of two components of 9:1 ratio in $CDCl_3$ solution. This ratio changes to *ca.* 2:1 in DMSO-d_6. The chemical shifts of the CH_3 and NH hydrogens in the two components are 3.15 and 6.45 ppm

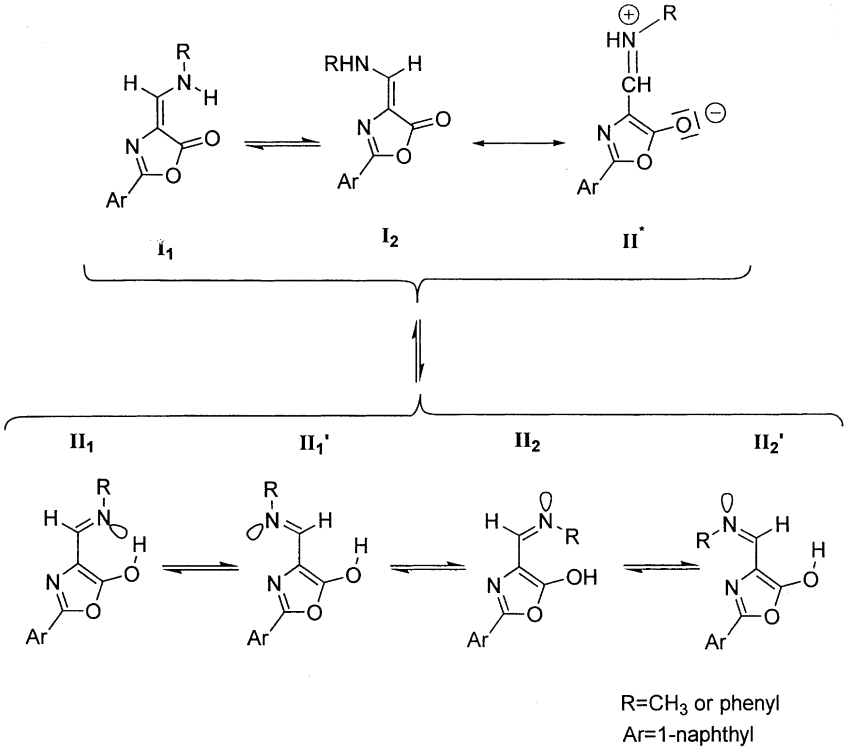

Figure 4. Isomer structures considered for assignment of 2-[1]-naphthyl-4-(alkylaminomethylene)-5(4H)-oxazolone derivatives.

Table 2. ^{1}H-NMR data of compounds **1b**, **2a–c** and **6b** in $CDCl_3$ solution at 500 MHz[a]

Compound	Component	CH_3 *d* (3H)[b]	=CH *d* (1H)[c]	H-2 ~*d* (1H)	H-3 ~*t* (1H)	H-4 ~*d* (1H)	H-5 ~*d* (1H)	H-6 ~*t* (1H)	H-7 ~*t* (1H)	H-8 ~*d* (1H)	NH broad
1b		1.45	7.37	8.20	7.47	7.94	7.84	7.52	7.62	9.32	4.43[d]
2a	major	3.15	7.31	8.18	7.52	7.94	7.88	7.54	7.60	9.35	6.45
	minor	3.59	7.32	8.15		7.92				9.40	5.80
2b		3.25 3.67	7.17	8.17	7.51	7.91	7.88	7.53	7.59	9.35	–
2c[e]	major	–	7.25	8.19	~7.55	7.98	7.90	~7.55	7.68	9.43	9.35
	minor		7.15	8.16		7.96				9.40	8.15
6b[f]	major	1.64	7.40	8.17		7.95	7.88			9.41	7.26[g]
	minor	1.67	7.36	8.16		7.92	7.89			9.44	

[a] Chemical shifts in ppm (δ_{TMS}=0 ppm). Assignments were supported by 2D-HSC (**1b**, **2a**, **2c** and **6b**) and 2D-COSY measurements (**1b**, **2a** and **6b**) and in the case of **2b** by DNOE experiments.
[b] Triplet for **1b**, $J(CH_3, CH_2)$: 7.3; two singlets for **2b**; doublet for all other compounds $J(CH_3, NH)$ or $J(CH_3, CH)$ for **2a** and **6b**, respectively, for both components: 5.1 (**2a**) and 7.2 (**6b**).
[c] *J*: 14.2 Hz (major component, **2a** and **6b**), 8.0 Hz (minor component, **2a** and **6b**); singlet for **1b** and **2b**.
[d] CH_2, quartet.
[e] Further signals (phenyl): H-2′,6′: 7.25 ~*d* (2H), H-3′,5′: 7.38 ~*t* (2H), H-4′: 7.16 ~*t* (1H).
[f] Further signals $C(sp^3)H$ ~*qi* (1H): 4.21 (major component), 5.37 (minor component).
[g] Double doublet, *J*: 14 and 7 Hz.

Table 3. ^{13}C-NMR chemical shifts (δ_{TMS}=0 ppm) of compounds **1b**, **2a–c** and **6b** at 125 MHz[a]

Compound	Component	CH_3	=CH	C-2	C-4	C-5	C-1	C-2	C-3	C-4	C-4a	C-5	C-6	C-7	C-8	C-8a
				←Oxazole ring→			←Naphthalene ring→									
1b		15.7	153.2	159.5	118.1	168.7	122.2	130.4	125.2	133.7	134.3	129.6	126.9	128.5	126.6	131.0
2a		35.9	144.7	155.4	109.9	167.8	123.1	129.2[b]	125.5	132.3	134.3	129.2[b]	126.7	127.9	126.6	130.7
2b		40.1 47.2	143.6	153.7	108.7	170.2	123.4	128.7	125.5	131.8	134.4	129.1	126.5[c]	127.8	126.6[c]	130.7
2c		–	134.5	156.3	113.1	168.2	122.9	129.4	125.3	132.6	134.2	129.1	126.8[c]	128.1	126.7[c]	130.7
6b	major	19.6	141.5	155.3	109.8	168.0	123.1	128.7	125.3	132.2	134.2	128.9	126.8	128.0	126.6	130.6
	minor	19.1	139.5		110.8	169.7				131.9			126.9		126.5	130.5

[a] Assignments were supported by DEPT for **1b**, **2a**, **2c** and **6b** by 2D-HSC and in the case of **1b** also by COLOC (HMBC) measurements. Further signals, CH_2 (**1b**): 73.0, phenyl (**2c**), C-1′: 139.8, C-2′,6′: 117.1, C-3′,5′: 130.2, C-4′: 124.8; C(sp^3)H (**6b**): 56.1 (major component), 53.4 (minor component), COOH: 173.7 (major component), 174.6 (minor component).
[b] Overlapping lines.
[c] Interchangeable assignments.

(major component) and 3.59 and 5.8 ppm (minor component). The large shift difference for the methyl group (0.44 ppm) is clear evidence for hindered rotation around the C(6)=N bond. The value of the NH resonance shifts excludes structures with chelation (I_1 and II_1). From these data one can conclude that I_2, II_1', II_2 and II_2' are the plausible variants for further consideration.

In the case of compound **2b** with the *N*,*N*-dimethyl substituent, the ^{1}H and ^{13}C spectra correspond to a stereohomogeneous product (Tables 2 and 3). All hydrogens and carbons give only one signals. However, the two methyl groups show separated ^{1}H- and ^{13}C-NMR signals, where the intensity ratio is 1:1 and the differences in chemical shifts are rather significant (0.42 and 7.1 ppm). These data refer to a mesomeric structure of tautomer type I (however, involving the predominance of a zwitterionic limiting form II^*). In order to determine the rotameric form we measured the NOE values resulting from saturating two methyl-group signals. In both experiments responses of the olefinic as well as of the H-8 naphthalene hydrogens were observed, but the ratio of the two DNOE signals were characteristically different. Namely, the irradiation of the upfield methyl signal resulted in 14.2 and 1.3% intensity enhancements of the =CH—N and H-8 signals, while saturating the downfield counterpart of the above *N*-methyl signal led to 5.9% and 5.2% responses. This means that the rotation around the C-6—N bond is hindered, but possible. In the solid phase, however the oxo-form is predominant as proven by high ν C=O IR frequency (1750 cm^{-1}) measured in a KBr disc.

The similar ^{1}H-NMR shift differences for the methyl signals in the isomers of **2a** and **2b** might indicate similar structures. Considering the strong steric interaction between the *N*-methyl and hydroxy groups in structure II_2 (Fig. 4), the two methyl signals of compound **2a** could be assigned to $II_1^{*\prime}$ and $II_2^{*\prime}$ structures, where the major component is $II_1^{*\prime}$. The predominance of $II_1^{*\prime}$ could be explained by steric hindrance between the oxazole ring and the *N*-methyl group in the $II_2^{*\prime}$ isomer. In accordance with this proposal the chemical shift of the *N*-methyl hydrogens is higher in the minor component ($II_2^{*\prime}$): the anisotropic neighbouring effect of the oxazole ring *N* results in the downfield shift of the $N(CH_3)$ signal. Another observation also underlines the relevance of the above supposition. We found that the vicinal CH–NH coupling for the major component involving the *trans*-arrangement of these hydrogens is 14.2 Hz, while it is only 8.0 Hz in the minor counterpart due to the *cis*-orientation of the interacting hydrogens.

Based on these observations and on the similar set of data obtained with **2c** and **6b** one can assume that the $II_1^{*\prime}$-type structure represents the predominant component of type **6** amino acid conjugates of **1b** observed during HPLC analyses.

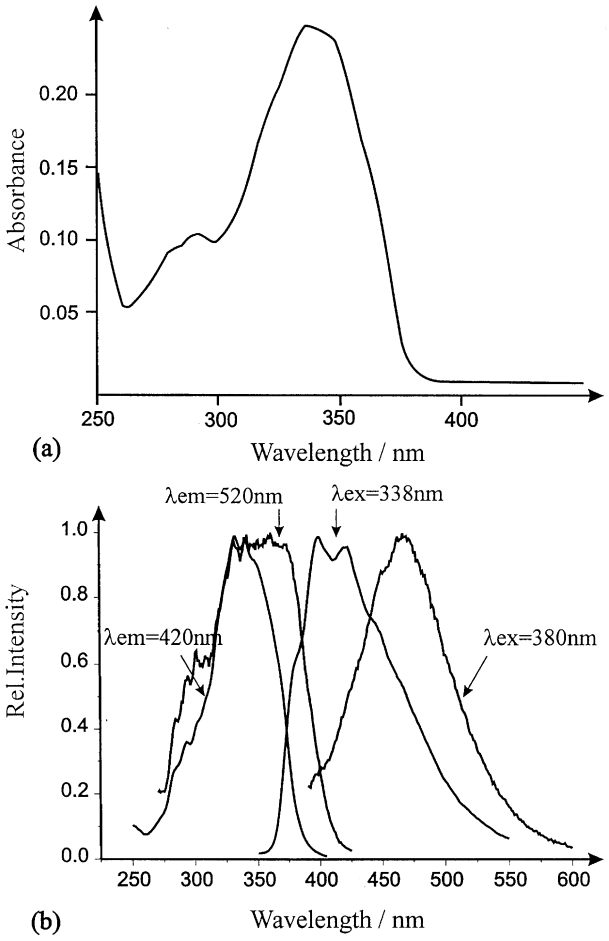

Figure 5. Absorption (a) and normalized emission and excitation (b) spectra of **1b** in ethanol (c=6.6×10^{-5} M) recorded at RT. Corresponding emission and excitation wavelengths are indicated in the figure.

2.3. UV and fluorescence spectroscopic characterization

UV and fluorescent spectroscopic properties of **1b** as well as compounds of type **6** were studied and the results are summarized in Figs. 5–7 and Table 4.

A typical absorption spectrum of **1b** is presented in Fig. 5(a). The highest molar extinction coefficient, 3.8×10^3 dm^3 mol^{-1} cm^{-1} can be measured at 338 nm in ethanol. When this solution of **1b** is excited at the absorption maximum, light emission can be recorded between 350 and

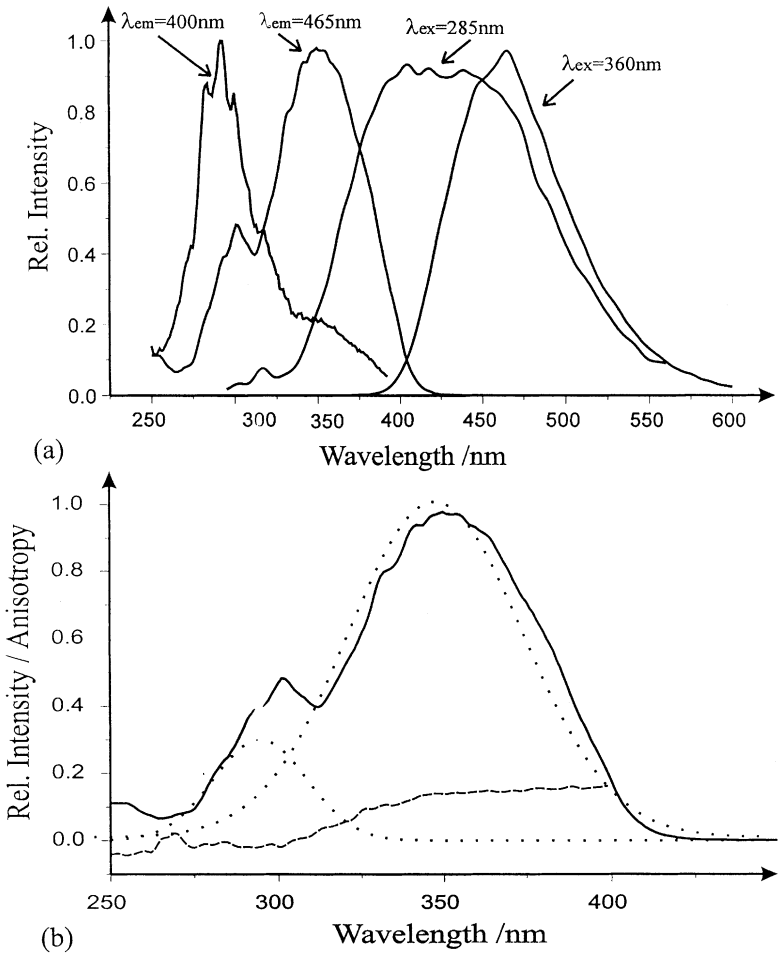

Figure 6. (a) Normalized emission and excitation spectra of compound **6b** recorded at RT in 0.14 M phosphate buffer, pH=7.4. Corresponding emission and excitation wavelengths are indicated in the figure. (b) Excitation anisotropy spectrum (dashed line) and normalized excitation spectrum (solid line) of compound **6b** (λ_{em}=465 nm) at RT in 0.14 M phosphate buffer, pH=7.4. Dotted lines represent the components of multiple Gaussian fitting.

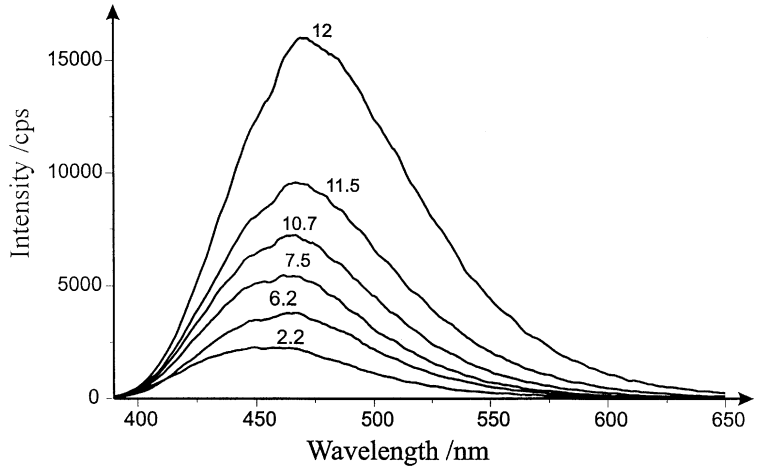

Figure 7. The pH dependence of emission spectra (λ_{ex}=360 nm) of compound **6b** in 0.1 M phosphate buffer.

Table 4. Characteristic spectroscopic properties of amino acid derivatives of **1b**

Compound		Absorption spectrum		Fluorescence spectrum	
Code	Amino acid	λ_{max} (nm)	ε ($dm^3\ mol^{-1}\ cm^{-1}$)[a]	λ_{ex} (nm)	λ_{em} (nm)[a,c]
6a	Gly	363.5	17700	380	472
6b	L-Ala	364.6	16700	352	472
6c	D-Ala	363.1	12400	395	490
6d	L-Leu×DCHA	368.9	19200	364	469
6e	D-Leu×DCHA	369.1	19800	372	466
6f	L-Ile	364.8	13200	352	472
6g	L-Pro	372.4	18000	376	463
6h	L-Phe	366.7	18400	356	472
6i	D-Phe	366.8	17700	356	472
6j	L-His	366.1[b]	n.d.[d]	368	478[b]
6k	L-Trp	370.9	21470	356	466
6l	α-Boc-L-Lys	365.0	17300	357	447

[a] Solvent: methanol, 0.01 mg mL^{-1}.
[c] Exciting at λ_{ex}.
[b] Solvent: acetonitrile, 0.01 mg mL^{-1}.
[d] No data.

600 nm [Fig. 5(b)]. The maxima of these broad emission bands are at 398 and 420 nm; furthermore two shoulders can be recognized around 380 and 460 nm. When the sample is excited at 338 nm, the relative quantum yield of fluorescence emission is 0.1. The lifetime of the **1b** excited state can be estimated as 2.1 ± 0.3 ns when $\lambda_{ex}=338$ nm and $\lambda_{em}=400$ nm.

The structure of the emission spectrum of **1b** obtained at $\lambda_{ex}=338$ nm could be indicative for more than one electronic transition. In order to elucidate this problem, a series of emission and excitation spectra were recorded at different excitation and emission wavelengths, respectively. When the wavelength of the observation was varied between 350 and 430 nm, excitation spectra of similar structure were recorded with a maximum centred at 330 nm. As an example the excitation spectrum belonging to $\lambda_{em}=420$ nm is presented in Fig. 5(b). This spectrum is slightly blue-shifted as compared to the absorption spectrum of the same compound. The obvious difference between the excitation and absorption spectra indicates the presence of two different electronic transitions (namely two chromophores). If the observation wavelength is shifted towards wavelengths longer than $\lambda=430$ nm, the excitation spectrum changes gradually with a redshift in its maximum from $\lambda=330$ nm to 360 nm. In Fig. 5(b) the excitation spectrum belonging to $\lambda_{em}=520$ nm is presented as an example. When the sample is excited at $\lambda=360$ nm or longer wavelengths, the maximum of the emission spectrum appears at $\lambda=465$ nm.

These data show that, both in the emission and excitation spectra of **1b**, two overlapping bands can be clearly distinguished. The maxima of the excitation spectra are around 330 and 360 nm and the corresponding emission maxima are at $\lambda=400$ and 465 nm. From the separation of the excitation and emission bands observed we can conclude that the sample emits light as a consequence of at least two electronic transitions. However, the amplitudes of the two emission bands are different (data not shown). Under similar conditions the emitted intensity is higher at $\lambda=400$ nm ($\lambda_{ex}=330$ nm) than at $\lambda=465$ nm ($\lambda_{ex}=360$ nm).

The absorption maxima and corresponding extinction coefficients of N^{α}-amino acid derivatives (**6a–l**) are listed in Table 4. The lifetime of the **6** excited state can be estimated as shorter than 1 ns when $\lambda_{ex}=338$ nm and $\lambda_{em}=400$ nm. The emission and excitation maxima of compounds of type **6** are presented in Table 4. Fig. 6(a) shows the fluorescence spectra of derivative **6b** in 0.14 M phosphate buffer, pH 7.4. Here and also in the other N^{α}-amino acid derivatives the two emission and excitation bands can be clearly recognized. The more intense emission band is centred at $\lambda=460$ nm ($\lambda_{ex}=360$ nm) and a weaker band can be detected at $\lambda=400$ nm ($\lambda_{ex}=285$ nm). In order to distinguish the excitation spectra of two different electronic transitions the excitation spectra of compound **6b** was deconvoluted by multiple Gaussian fitting. The result is presented in Fig. 6(b). The best fit can be received as a sum of two Gaussian components, the maxima of these components show a very good agreement with the two excitation maxima of **6b** [see Fig. 6(a)].

The short fluorescence lifetime of compound **6b** makes possible the measurement of its excitation anisotropy spectrum even in diluted solution (0.14 M, pH 7.4). In the anisotropy spectrum [Fig. 6(b)] two wavelength ranges can be distinguished. Between $\lambda=250$ nm and 320 nm negative anisotropy (-0.05 at 285 nm) was observed, while above $\lambda=320$ nm positive anisotropy (0.12 at 350 nm) could be detected. These two wavelength regions fit well to the recorded [Fig. 6(a)] and calculated excitation bands [Fig. 6(b)]. The result of mathematical spectral analysis and the excitation anisotropy spectrum confirms that the two electron transitions belong to two different chromophores.

Based on the observations described above it cannot be excluded that the two electron transitions belong to the two isomers. To investigate this possibility further spectroscopic studies were performed at different temperatures between 15°C and 50°C. As mentioned before we found that the ratio of the isomer composition changed with temperature during the HPLC analysis. If the two electron transitions belong to two geometric isomers, the relative

probability and consequently the relative amplitude of the emission and excitation maxima have to be dependent on the temperature. The structures of the emission and excitation spectra of compound **1b** and compound **6b** were always similar to the spectra presented in Figs. 5(b) and 6(a), regardless of the temperature. From these results we can conclude that the isomers of molecule **1b** both have two independent electronic transitions (chromophores).

The emission spectra of compound **6b** were recorded also in 0.1 M phosphate buffer solutions of various pH values. As presented in Fig. 7, the amplitude of the emission band centred at λ=465 nm varies according to the pH value of the solution. The pH-sensitivity was especially marked above pH=10. These changes in the emission intensity might be correlated with the ionization of the carboxy group of the alanine moiety. At pH 2.2 it could be fully protonated, while at higher pH values this group is anionic. However, pronounced changes observed above pH 10 are indicative of deprotonation of the oxazole ring nitrogen at high pH or of a more complex set of interactions occuring on elevation of the pH. In contrast, the light intensity detected at 400 nm (λ_{ex}=285 nm) was independent of the solvent pH (data not shown). The pH dependence of the main electron transition of the N^{α}-amino acid conjugate indicates that this molecule or other amino acid derivatives of 4-ethoxymethylene-2-[1]-naphthyl-5(4*H*) oxazolone can be useful indicators of the pH of their environment.

3. Conclusion

A simple and general method has been devised to prepare conjugates between the oxazolone **1b** and amine and amino acid by direct reaction with the corresponding amino component. An efficient synthesis of **1b** is also reported. Physico-chemical as well as spectroscopic properties of **1b** and its amino acid derivatives indicate that these compounds could be useful for labelling proteins, glycoproteins or peptides with a new fluorophore possessing intense and pH-sensitive emission characteristics. The pH-sensitive feature of the chromophore should be emphasized and could be advantageous to monitor intracellular movement of compounds in cellular compartments with different pH values. **1b** could be introduced directly to biomolecules possessing a free amino group, while the amino acid derivatives–according to preliminary experiments–could be utilized as building blocks for solid-phase peptide synthesis.[14] In addition, the new, hapten-analogue amino acids might be investigated in down-regulation of contact sensitivity.[15]

4. Experimental

4.1. General

Amino acids and solvents were obtained from Reanal, Budapest, Hungary, while other reagents were from Aldrich and were used without further purification. TLC analyses were performed on Merck 5553 Kiesgel 60, 0.2 mm silica plates (Darmstadt, Germany). For the development the following eluents were used: pyridine/acetic acid/H_2O/EtOAc (20:6:11:333, v/v/v/v) (system A), *n*-butanol/acetic acid/H_2O (4:1:1, v/v/v) (system B), chloroform (system C) and *n*-hexane/EtOAc (3:1, v/v) (system D). Spots were detected by I_2, Cl_2/toluidine or by UV and/or fluorescence light. RP-HPLC analyses were carried out on a Waters (Milford, MA, USA) HPLC system using a C_{18} ODS column (3.9×150 mm; packed with spherical 10 μm silica with 300 Å pore size). The gradient elution was developed using 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile/water=80/20 v/v as eluent B. During the analysis the content of eluent B was between 5% and 60%, in 30 min (system I) or 40–80% in 30 min (system II). The flow rate was 1 mL min^{-1} at RT. The samples were dissolved in eluent B and 20–50 μL of solution was injected. Peaks were detected λ_1=214 nm and λ_2=360 nm. FAB-MS spectra were obtained on a VG-ZA-2SEQ spectrometer (Fisons, UK), and elemental analyses (C, H, N) were performed on a Hereaus CHN-O Rapid (Hereaus Gmbh, Germany) apparatus. IR spectra were recorded on a Specord IR Spectrophotometer (Zeiss, Germany). The ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 solution in 5 mm tubes at RT, on a Bruker DRX 500 spectrometer at 500.13 (^{1}H) and 125. 76 (^{13}C) MHz, using the deuterium signal of the solvent as the lock and TMS as internal standard. The standard Bruker microprogram NOEMULT.AU was used to generate NOE. DEPT spectra were run in a standard manner, using only the Θ=135° pulse to separate CH/CH_3 and CH_2 lines phased 'up' and 'down', respectively. The 2D-HSC spectra were obtained by using the standard Bruker pulse program HXCO.AU. UV–Vis and fluorescence spectra were routinely registered on a Perkin–Elmer Lambda 2S spectrophotometer and on a Hitachi F-4500 spectrofluorimeter, respectively.

4.1.1. Preparation of 1-naphthoyl-glycine (3). Seventy-five grams (1.0 mol) glycine was dissolved in 1000 mL of 1.0 M NaOH solution (1.0 mol), and 500 mL dioxane was added. From separate dropping funnels 151 mL (191 g, 1.0 mol) 1-naphthoyl-chloride and 500 mL 2.0 M NaOH solution (1.0 mol) were added in 3 h at the same rate using efficient mechanical stirring. The stirring was continued at RT for a further 4 h. Dioxane was removed in vacuo, and the resulting solution was extracted 5 times with 100 mL of EtOAc. The aqueous phase was acidified with 5.0 M HCl to pH 2. The precipitated product was filtered off, washed with water and dried. This can be used for the synthesis of **1b** without further purification, or can be recrystallized from water. Mp: 152°C [148–149°C[11]]. Yield: 195g (85%).[11]

4.1.2. Synthesis of 4-ethoxymethylene-2-[1]-naphthyl-5(4*H*)-oxazolone (1b). A solution of 1-naphthoyl-glycine (16.4 g, 71.6 mmol), acetic anhydride (67.3 mL, 716 mmol) and triethyl orthoformate (35.7 mL, 215 mmol) in EtOAc (100 mL) was refluxed vigorously for 3 h. The product was precipitated with *n*-hexane (200 mL) and kept at 4°C in a refrigerator overnight. The crystalline crude product **1b** was filtered off, dried in vacuo and recrystallized twice from MeOH to give 11.3 g (42.3 mmol, 59%) of pale yellow crystals. Mp: 131–132°C; TLC: R_f=0.80 (system C). MS (FAB): *m*/*z*=268.0 (MH^+, 62), 254.0(14), 171.0(54), 155.0(80), 127.0(43), 93.1(100). Anal. calculated for

$C_{16}H_{13}NO_3$: C 71.90, H, 4.90, N 5.24. Found C 71.89, H, 4.92, N 5.24. NMR data are presented in Tables 2 and 3.

4.1.3. Synthesis and purification of amino acid derivatives of 1b (6a–6k). General procedure. 4-Ethoxymethylene-2-[1]-naphthyl-5(4*H*)-oxazolone **1b** (0.5 g, 1.87 mmol) and the corresponding amino acid (**5**) (1.87 mmol) were suspended in MeOH (20 mL) with vigorous stirring. The reaction proceeded at RT for 24 h. Work-up procedures (A–D) started with removal of MeOH in vacuo and continued as outlined below. Compounds **6d** and **6e** were isolated as dicyclohexylamine salts. Procedure A: product was triturated and washed with water (20 mL) (L-Ile, L-Trp), with Et_2O (L-Pro) or with Et_2O and water (Gly, L-Phe, D-Phe, His), filtered off and dried in vacuo. Procedure B: product was dissolved in Et_2O (20 mL), precipitated with *n*-hexane, filtered off, dried, suspended in water, filtered off, washed with water (L-Ala, D-Ala). Procedure C: product was dissolved in Et_2O (20 mL), cooled with ice, DCHA (375 mL, 1.87 mmol) was added, the precipitated product was filtered off, washed with Et_2O (L-Leu, D-Leu). Procedure D: product was recrystallized from 70% methanol–water. After isolation products were dried over P_2O_5 in vacuo. All products are yellowish and are crystalline. Yields, melting point and R_f values are summarized in Table 1. Chemical characteristics of compounds **6a–l** are presented in Table 1. For NMR data of compound **6b** see Tables 2 and 3.

4.1.4. Synthesis of 4-(methylaminomethylene)-2-[1]-naphthyl-5(4*H*)-oxazolone (2a) and 4-(di-methylaminomethylene)-2-[1]-naphthyl-5(4*H*)-oxazolone (2b). 1b (0.5g, 1.87 mmol) was dissolved in CH_2Cl_2 (10 mL) and 0.3 M methylamine in CH_2Cl_2 solution (**2a**, 31 mL) or 0.3 M dimethylamine in CH_2Cl_2 solution (**2b**, 31 mL) was added. The mixture was stirred at RT for 4 h. Volatile components were removed in vacuo and the product was precipitated with water (20 mL). After filtration and washing with water (3×5 mL) and MeOH (1×5 mL) the product was dried over P_2O_5 in vacuo.

2a: yield=65%, mp 152–153°C, R_f=0.88 (system B), R_t=13.4 min (isomer 1) and 19.5 min (isomer 2) (system II). For ^{1}H- and ^{13}C-NMR data see Tables 2 and 3. Anal. calculated for $C_{15}H_{12}N_2O_2$: C 71.42, H, 4.79, N 11.10. Found C 71.46, H, 4.76, N 11.18.

2b: yield=92%, mp 173–174°C, R_f=0.82 (system B), R_t=19.4 min (system II). For ^{1}H- and ^{13}C-NMR data see Tables 2 and 3. Anal. calculated for $C_{16}H_{14}N_2O_2$: C 72.16, H, 5,30, N 10.52. Found C 72.21, H, 5.22, N 10.55.

4.1.5. Synthesis of 2-[1]-naphthyl-4-(phenylaminomethylene)-5(4*H*)-oxazolone (2c). 1b (73 mg, 0.27 mmol) was dissolved in a mixture of MeOH (1 mL) and CH_2Cl_2 (1 mL). Freshly vacuum-distilled aniline (100 μL, 1.1 mmol) was added to the solution. The mixture was stirred at RT for 18 h. Volatile compounds were removed in vacuo and the product was dried over P_2O_5.

Yield=90%, mp 172–173°C, R_f=0.51 (system D), R_t=12.9 min (isomer 1) and 18.3 min (isomer 2) (system II). For ^{1}H- and ^{13}C-NMR data see Tables 2 and 3. Anal. calculated $C_{20}H_{14}N_2O_2$: C 76.42, H, 4.49, N 8.91. Found C 76.36, H, 4.44, N 8.85.

4.2. Singlet state studies

Ground-state absorption spectra were recorded by use of a Cary 4E spectrophotometer. Molar absorption coefficients (ε) were obtained from the slope of the linear part of Beer–Lambert plots of absorbance versus concentration. Corrected steady-state emission and excitation spectra were obtained using an FS900CD spectrofluorimeter (Edinburgh Analytical Instruments, UK) with Xe lamp excitation and Hammamatsu photomultiplier (R955) detection. Emitted fluorescence intensity was measured at an angle of 90° relative to the exciting light. Resolution of monochromators was 0.5 nm. The fluorescence anisotropy was defined as

$$A = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are observed intensities measured with emission polarizers parallel and perpendicular to the vertically polarized exciting beam, respectively. G is a wavelength-dependent factor used to correct for the inability of the instrument to transmit differently polarized light equally. For the fluorescence lifetime (τ_F) measurements the same instrument was used with a hydrogen-filled flash-lamp excitation with 1.5 ns pulse width and emission decays were determined by a single-photon counting method. Air-saturated samples were excited at the corresponding excitation maxima at RT. During the absorption and fluorescence measurements the samples were kept at RT. The sample concentration was 10^{-5} M. If the absorbance of the sample exceeded 0.05 the observed fluorescence intensity was corrected for the inner filter effect of the solution. The fluorescence quantum yields of derivatives (Φ_F^S) in different media were estimated as

$$\Phi_F^S = \left(\frac{n^S}{n^R}\right)^2 \cdot \frac{F^S}{A^S} \cdot \frac{A^R}{F^R} \cdot \Phi_F^R \quad (2)$$

where Φ_F^R is the quantum yield of rhodamine B in ethylene glycol as reference compound,[16,17] A is the absorbance at the wavelength of the excitation, F is the integrated area of emission spectra, n is the refractive index of the solution, the (S) superscript refers to the sample and (R) to the reference. The integrated fluorescence of the sample was compared to that of the standard after adjusting concentrations so that OD values of the sample and reference at the excitation wavelength were equal and smaller than 0.05.

Acknowledgements

These studies were supported by grants from the Hungarian Ministry of Education (FKFP 1191/1997 [GCs, EB]) and from the Hungarian Research Fund (OTKA) No. T-021120, No. T-03838.

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