Fluorescent Amino Acids

A Very Short Route to Enantiomerically Pure Coumarin-Bearing Fluorescent Amino Acids**

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Fluorescent derivatives of biologically active peptides are useful experimental tools for studying biological structure and function[1] and for visualizing intracellular processes or molecular interactions.[2] To achieve adequate detection of peptide fluorescence, the monitored peptide must either include a natural fluorescent amino acid, such as tyrosine (Tyr) or tryptophan (Trp), or must be labeled with extrinsic or intrinsic probes.[3] Since C- and N-terminal domains are often important interactive sites, the incorporation of the fluorescent label into the peptide at a well-chosen position is critical to avoiding changes in the conformational and biological characteristics of the parent peptide.[4a] The use of an amino acid as a fluorescent chromophore[4] offers at least two advantages: first, peptides containing this amino acid can be synthesized by solid-phase peptide synthesis (SPPS); and second, since both ends of these peptides are free, other residues may be attached to improve solubility or lability.[5] In addition to the excitation and emission properties of a

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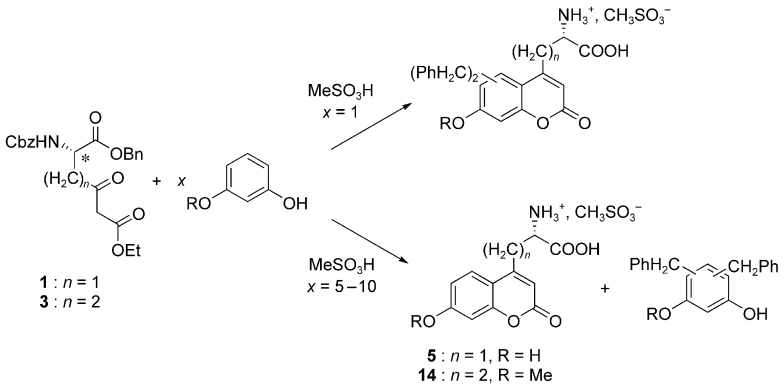
fluorescent amino acid its steric hindrance must also be considered. Thus, augmenting the conjugation in an amino acid increases its fluorescence-emission properties. On the other hand, however, such increased conjugation (as in the case of polycycles) gives rise to more steric hindrance, associated with lowered coupling yields. Furthermore, since a higher chemical stability is expected if the fluorophore is linked to the amino acids by a side-chain carbon–carbon bond, we have focused on developing small and sensitive fluorescent coumaryl amino acids that could be incorporated efficiently into peptides in place of Tyr or Trp.

Coumarins are interesting fluorescent molecules due to their relatively high quantum yields, low bleaching, reasonable stability, and relative ease of synthesis. They have been used extensively to label amines, thiols, and acids,[6] thereby providing useful experimental tools.[7] To date, enantioselective synthesis of amino acids,[8] especially coumaryl amino acids, has been carried out by diastereoselective alkylation of chiral glycine equivalents. For example, the chiral auxiliaries of Oppolzer et al.[9] and Williams[10] were used for the synthesis of L-(6,7-dimethoxycoumar-4-yl)alanine and L-(7-methoxycoumar-4-yl)alanine (Mca, (*S*)-**6**), respectively.[4c,d] These methods produced optically pure amino acids, but they required multiple steps and the amino acids were obtained in poor global yields. Herein we report a more rapid, cheap, and versatile synthesis of coumaryl amino acids that led to coumaryl alanines and coumaryl ethylglycines in either a two- or a three-step process.

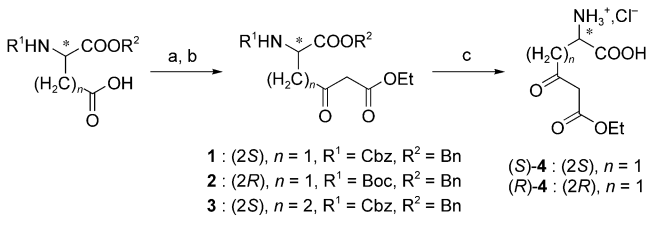
Protected aspartic (Asp) and glutamic acids (Glu) were used as chiral starting materials,[11] thus avoiding high-cost chiral auxiliaries. They were transformed into side-chain β-ketoesters (which we had chosen as coupling partners for the condensation with phenols to obtain the coumarin moiety[12]) by using the procedure of Masamune et al.[13] (Scheme 1). Pure β-ketoesters **1**–**3** were obtained in good yields after silica-gel column chromatography.

Coumarins have been synthesized by many pathways, including von Pechmann, Perkin, Knoevenagel, Reformatsky, and Wittig reactions.[14] The von Pechmann reaction is one of the simplest and most straightforward methods. It involves a phenol condensation with a β-ketoester in the presence of a variety of Brønsted[14a,15] and Lewis acids.[12,16] We chose the von Pechmann condensation in methanesulfonic acid (MSA).[15] This condensation, carried out in the presence of 25 equivalents of MSA, proceeded smoothly and resulted in the complete dissolution of starting material and the complete conversion of the β-ketoester. Reaction times depended on the substituents on the phenol ring: the greater the number of electron-donating groups, the shorter the reaction time.

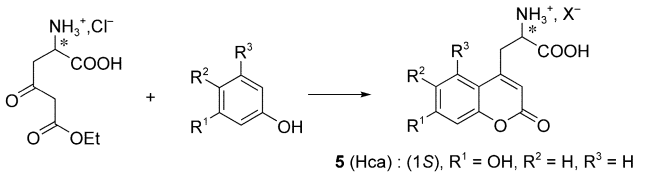
Since OCO–benzyl and NCO_2–benzyl bonds are not stable under these conditions, running the reaction in the presence of one equivalent of resorcinol produced polybenzylated coumarins. This could be avoided by using a large excess of the resorcinol derivative, which acts as a scavenger for the benzyl cations (Scheme 2). This allowed the formation of compounds **5** and **14** in only one, direct step. For expensive resorcinols, we first deprotected both amine and carboxy groups to avoid side reactions (step c in Scheme 1). The deprotected aspartic acid derived β-ketoester, (*S*)- or (*R*)-**4**, was then treated with various polysubstituted phenols in the presence of MSA to give compounds **5**–**13** (Scheme 3). Hydrogenolytic conditions used for Asp are unsuitable for



Scheme 2. General synthesis of coumaryl amino acids.



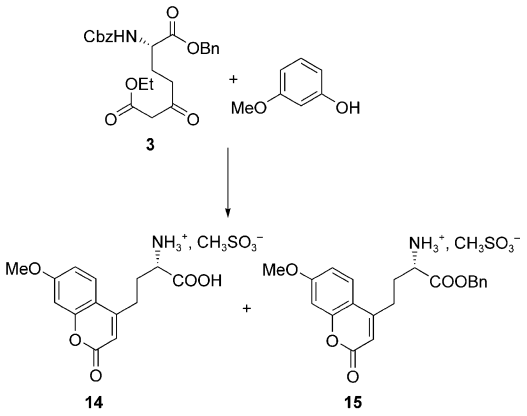
Scheme 1. Synthesis of amino acid derived β-ketoesters. a) Carbonyldiimidazole, THF, room temperature; b) magnesium salt of monoethyl malonic acid, THF, 0 °C and 12 h at room temperature, 50–80%; c) H_2, Pd/C 10%, HCl, AcOEt/EtOH, room temperature, 90–100% (and for **2**, 1:1 TFA:$CHCl_3$ at 0 °C and 1 h at room temperature). TFA = trifluoroacetic acid, Cbz = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Bn = benzyl.



5 (Hca) : (1*S*), R^1 = OH, R^2 = H, R^3 = H
(*S*)-**6** (Mca) : (1*S*), R^1 = OMe, R^2 = H, R^3 = H
(*R*)-**6** : (1*R*), R^1 = OMe, R^2 = H, R^3 = H
7 : (1*S*), R^1 = OMe, R^2 = OMe, R^3 = H
8 : (1*S*), R^1 = OH, R^2 = Cl, R^3 = H
9 : (1*S*), R^1 = OEt, R^2 = H, R^3 = H
10 : (1*S*), R^1 = OMe, R^2 = H, R^3 = OH
11 : (1*S*), R^1 = OH, R^2 = H, R^3 = OMe
12 : (1*S*), R^1 = OMe, R^2 = H, R^3 = OMe
13 : (1*S*), R^1 = OH, R^2 = H, R^3 = OH

Scheme 3. Synthesis of aspartic acid derived coumaryl amino acids. MSA (25 equiv), 0 °C, then room temperature, 2–4 h, 16% (**8**) to 71% (**6**); $X^- = CH_3SO_3^-$ ((*S*)-**6**) or $CF_3CO_2^-$ in case of purification by semi-preparative HPLC on a Kromasil C_8 column (A (H_2O+0.1% TFA) and B (70:30 CH_3CN:H_2O+0.09% TFA), with an appropriate linear gradient of B).

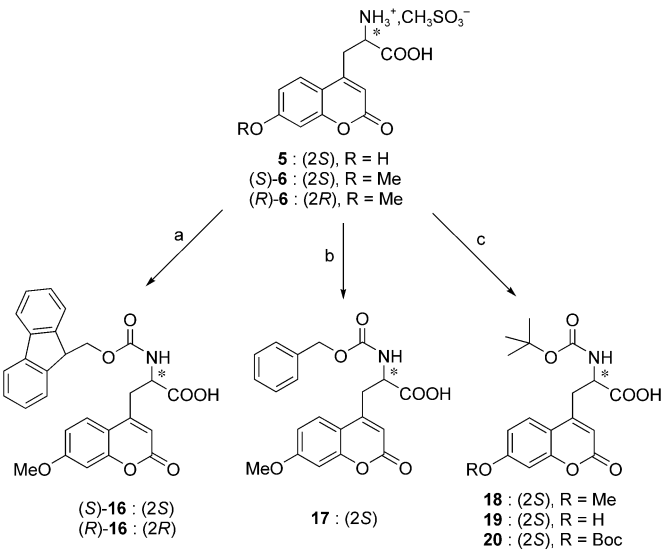
Glu.[17] Therefore, coumarin formation was conducted on the still-protected β-ketoester **3** with a large excess of 3-methoxyphenol (Scheme 4), which led to two products. In one product, compound **14**, the amine and acid functionalities were



Scheme 4. Synthesis of glutamic acid derived coumaryl amino acids. 3-Methoxyphenol (10 equiv), MSA (25 equiv), 0 °C then room temperature, 2 h, 60% (ratio **14**:**15** = 62:38).

deprotected, whereas the other, compound **15**, still bore the benzyl ester group. Coumaryl amino acids in which the amino group was protected with a widely used protecting group, such as Fmoc ((*S*)- and (*R*)-**16**), Cbz (**17**), or Boc (**18**–**20**), were also synthesized (Scheme 5).

Since a stereogenic carbon atom is present in these amino acids, we measured optical rotation (Table 1) and also determined the enantiomeric purity of one of the compounds. We assumed that the other compounds would exhibit the same sensitivity towards racemization, given that the most probable source of racemization should be the earlier formation of the β-ketoester in a slightly alkaline medium. To verify the optical purity, the amino groups of (*S*)- and (*R*)-**6** were protected with an Fmoc group and the compounds coupled with *tert*-butylalaninate. The purity of the corresponding diastereomeric products **21** and **22** was examined by



Scheme 5. Synthesis of *N*-protected coumaryl amino acids. a) 10% $NaHCO_3$ at 0 °C, FmocCl at 0 °C, 1:1 dioxane:H_2O, 1 h at 0 °C then 2 h at room temperature, 80% ((*S*)-**16**) and 70% ((*R*)-**16**); b) NaOH at 0 °C, CbzCl at 0 °C, 1:1 dioxane:H_2O, 1 h at room temperature, 91%; c) Boc_2O at 0 °C, 1:1 dioxane:5% $NaHCO_3$, 1 h at 0 °C then 3 h at room temperature, 84% (**18**) and 75% (**19** and **20**). Fmoc = 9-fluorenylmethoxycarbonyl.

using appropriate HPLC isocratic conditions (Figure 1). In the case of **21**, only one diastereomer could be detected. For **22**, a diastereomeric ratio of 98:2 was observed for the crude reaction mixture. We can conclude that no racemization occurred during the β-ketoester formation, in the course of the cyclization, or during the protection and coupling processes (BOP (= benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate)/DIEA (= *N*,*N*-diisopropylethylamine)). These observations suggest that the *N*-protected amino acids obtained can be incorporated by SPPS into peptides without risk of racemization.

Absorption and fluorescence wavelength maxima, extinction coefficients, and fluorescence quantum yields (QY) were measured for each compound (Figure 2, Table 1). The optical

Table 1: Physicochemical properties of coumaryl amino acids.[a]

		n	R^1	R^2	R^3	Yield [%]	$[\alpha]_D^{20}$[a]	$\lambda_{Abs.}$ [nm][b]	$\lambda_{Em.}$ [nm][b]	ε [cm^{-1} M^{-1}][c]	QY[d]
Trp	–	–	–	–	–	–	–	278[18a]	352[18a]	5300[18a]	0.12[18b]
Tyr	–	–	–	–	–	–	–	274[18a]	303[18a]	1400[18a]	0.13[18b]
5	(2*S*)	1	OH	H	H	47	8.4	329	464	9000	0.49
(*S*)-**6**	(2*S*)	1	OMe	H	H	71	12.9	324	383	12000	0.18
(*R*)-**6**	(2*R*)	1	OMe	H	H	70	−12.1	323	386	14000	0.15
7	(2*S*)	1	OMe	OMe	H	45	n.d.[e]	345	423	12000	0.60
8	(2*S*)	1	OH	Cl	H	16	3.2	331	465	10300	0.54
9	(2*S*)	1	OEt	H	H	39	15.7	325	387	13300	0.17
10	(2*S*)	1	OMe	H	OH	36	n.d.[e]	329	421	6800	0.50
11	(2*S*)	1	OH	H	OMe	18	10.1	323	421	12700	0.18
12	(2*S*)	1	OMe	H	OMe	34	12.0	324	419	11500	0.39
13	(2*S*)	1	OH	H	OH	48	13.2	327	463	11800	n.d.[e]
14	(2*S*)	2	OMe	H	H	37	19.4	321	380	12500	0.21
15	(2*S*)	2	OMe	H	H	23	n.d.[e]	322	381	14600	0.17

[a] Determined in 1 N HCl. [b] Determined in 95% ethanol. [c] Extinction coefficient. [d] Quantum yield using quinine sulfate in sulfuric acid as a standard reference. [e] Not determined.

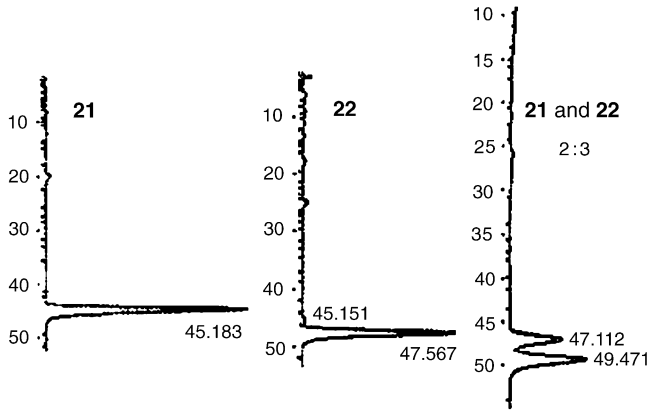


Figure 1. HPLC chromatograms of diastereoisomers **21** and **22**. HPLC isocratic conditions on a Vydac C_{18} column (38% A (H_2O + 0.1% TFA) and 62% B (3:1:1 CH_3CN:EtOH:MeOH)).

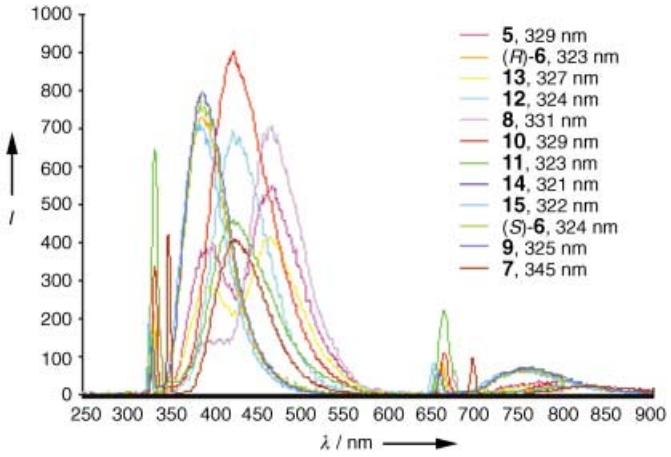


Figure 2. Fluorescence properties of the coumaryl amino acids **5**–**15**.

properties of the natural fluorescent amino acids Trp and Tyr were included for comparison. The novel coumaryl amino acids **5**–**14** had higher wavelength values and higher absorption values than Trp or Tyr, and they displayed higher quantum yields than Trp or Tyr, especially in the case of compounds **5**, **7**, **8**, **10** and **12**, with values ranking from 0.39 to 0.60 versus 0.12 and 0.13 for Trp and Tyr, respectively. These characteristics make compounds **5**–**14** good fluorophores for biological studies.

The use of such fluorescent amino acids in SPPS was validated by adding *N*-Fmoc-protected (*S*)-**6** (Mca) to the C-terminal end of penetratin, a 16 amino acid peptide corresponding to the third helix of the homeodomain of the Antennapedia protein (to give peptide 1, see Experimental Section).[19] To investigate whether the coumaryl amino acid could be used in biological studies, compound **5** (Hca) was added to the N-terminal end of penetratin (to give peptide 2, see Experimental Section). After incubation of this peptide in HeLa cells, we visualized its internalization by confocal microscopy (Figure 3), thus validating the use of coumaryl

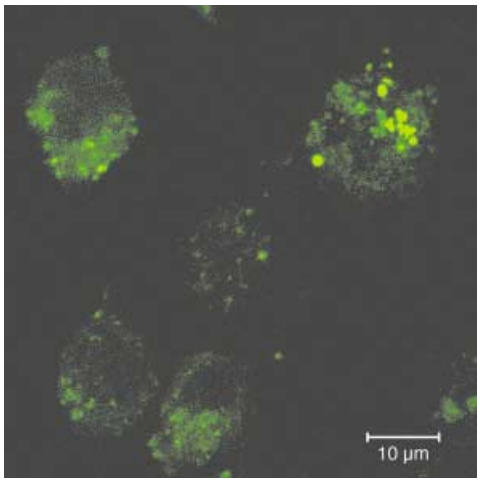


Figure 3. Peptide visualization using confocal microscopy. HeLa cells grown in DMEM (= Dulbecco's minimum essential medium) were treated with 10 μM peptide 2. After 2 h incubation at 37 °C, the cells were washed with PBS (= phosphate-buffered saline), fixed with 3% PFA (= paraformaldehyde) for 20 min at room temperature and mounted for examination. Images were obtained with a Zeiss LSM510 confocal scanning microscope using an argon ion laser set at 488 nm.

amino acids as intrinsic fluorescent labels.

peptide 1 H_2N–RQIKIWFQNRRMKWKK–Mca–COOH
peptide 2 H_2N–Hca-RQIKIWFQNRRMKWKK–COOH

In conclusion, we have developed an extremely direct synthesis of optically pure fluorescent amino acids, starting from inexpensive chiral materials. Our synthesis represents a useful tool in the preparation of coumaryl amino acids, which can be easily incorporated into peptides and used in biological studies. Potential applications of these molecules include the wide range of investigating cellular processes and other aspects of biological mechanism and function.

Experimental Section

Synthesis of peptides 1 and 2: SPPS by using small-scale Fmoc chemistry on a HMP (= 4-hydroxymethylphenoxy) resin, DCC/HOBt coupling method (BOP/HOBt/DIEA for Hca), removal of Fmoc groups with piperidine (20% in NMP), resin cleavage with TFA/TIPS/H_2O (9.5:0.25:0.25 in volume) and HPLC semipreparative purification on a Vydac C_{18} column (A (H_2O+0.1% TFA) and B (70:30 CH_3CN:H_2O+0.09% TFA), using a linear gradient of B from 10 to 90% in 30 min). DCC = *N*,*N*′-dicyclohexylcarbodiimide, HOBt = 1-hydroxybenzotriazole, NMP = *N*-methylpyrrolidinone, TIPS = triisopropylsilane.

See Supporting Information for detailed reaction conditions and compound characterization.

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