Synthesis and Solid-Phase Application of a 9-Xanthenyl Handle

Csaba Somlai[a], Péter Hegyes[b], Róbert Fenyö[a], Gábor K. Tóth[a], Botond Penke[a], and Wolfgang Voelter[c]

[a] University of Szeged, Department of Medical Chemistry, Dóm tér 8, H-6720 Szeged, Hungary
[b] University of Szeged, Department of Organic Chemistry, Dóm tér 8, H-6720 Szeged, Hungary
[c] Abteilung für Physikalische Biochemie, Physiologisch-chemisches Institut der Universität Tübingen, Hoppe-Seyler-Strasse 4, D-72076 Tübingen, Germany

Reprint requests to Dr. C. Somlai.
Fax: (36) 62-545 971. E-mail: scsaba@mdche.szote.u-szeged.hu

Z. Naturforsch. **56b**, 526–532 (2001); received November 2, 2000

Polymer Support, Solid-Phase Synthesis, Xanthene

The acid-labile 5-[(9-aminoxanthen-2-yl)oxy]valeric acid was prepared in a six-step route. The usefulness of the resulting handle was investigated with solid-phase peptide synthesis of cholecystokinin-8 sulfate and the phosphorylated fragment of the ζ-subunit of the T-cell receptor complex 138–144.

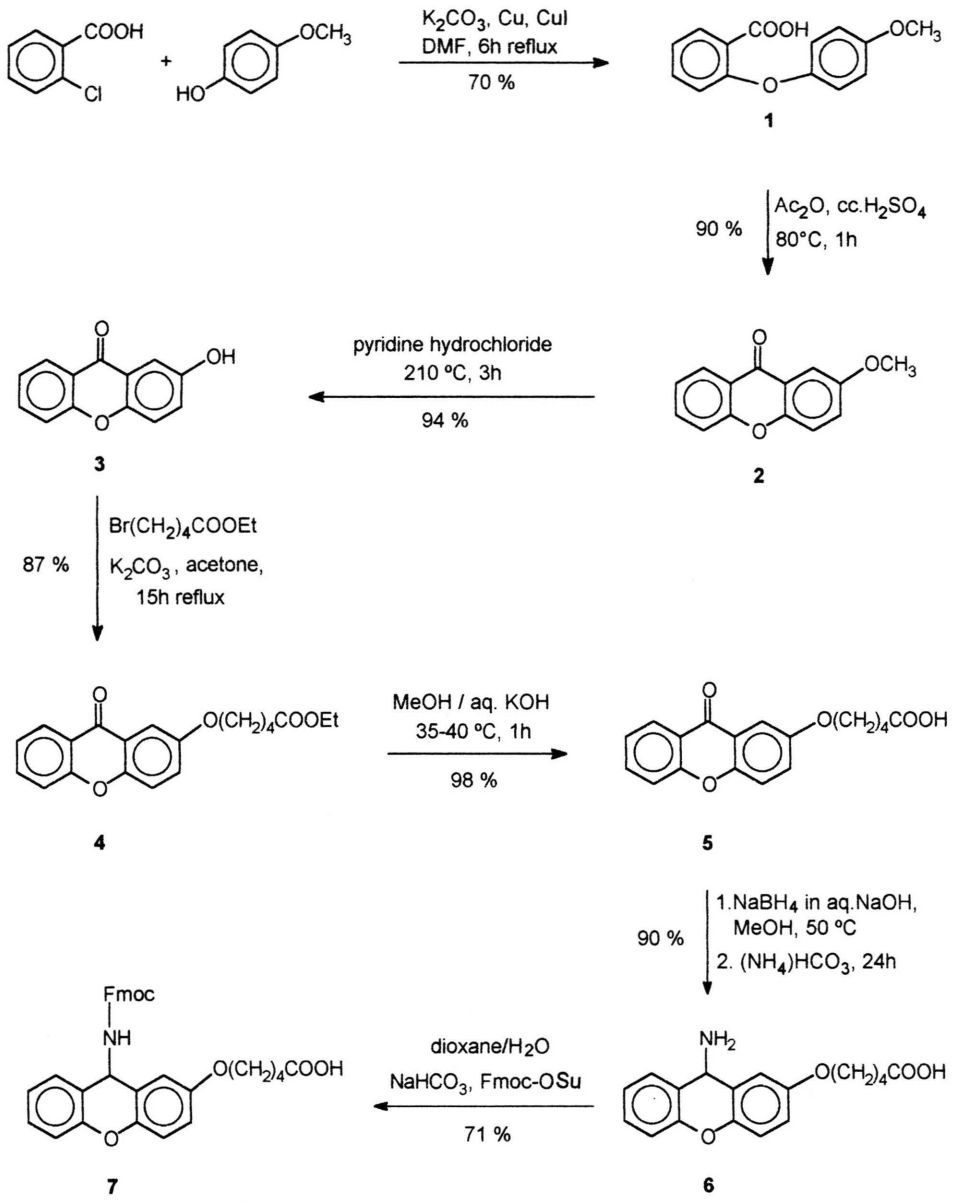
Introduction

Until now, a series of polymers and handles have been used in the solid-phase peptide synthesis [1–10] to obtain acid-sensitive peptides and/or partially or fully protected peptide fragments. Protected peptide amides can be released from xanthenyl-modified resins under very mild conditions without the risk of premature side-chain deprotection [11–13]. Earlier studies on the preparation and the application of different xanthene-derived compounds as handles for solid-phase peptide synthesis [11–14] prompted us to try the large-scale synthesis of 5-[(9-aminoxanthen-2-yl)oxy]valeric acid (XAL_2) and to reinvestigate its efficiency in this field. On the other hand, we reported in 1994 [14] that semiempirical quantumchemical methods (AM1, MNDO, PM3) and programs (AMPAC, MOPAC, PcMOL) may be useful tools to design new acid-sensitive handles [14–16]. According to our results, there is a close relationship between the calculated enthalpy and free enthalpy values of protolytic cleavage reactions and the experimentally measured acid-labilities of the compounds. As a result 5-[(9-hydroxyxanthen-3-yl)-oxy]valeric acid (XAL_3) was selected and synthesized, and its acid-lability was checked using 5% TFA in CH_2Cl_2. As the final reduction step of this synthesis as well as the isolation of the end-product met with some difficulties and required care (easy loss of the 9-hydroxy functional group), preparation of the appropriate 2-substituted compound (XAL_2) proved to be much easier and less risky [13].

Results and Discussion

Based in part on our experience [14] and in part on a reasonable literature procedure [13], the desired target compound, 5-[(9-aminoxanthen-2-yl)-oxy]valeric acid (XAL_2) was prepared on a six step route (Scheme 1) starting from *o*-chlorobenzoic acid and 4-methoxyphenol applying the Ullmann reaction [17]. The diphenyl ether derivative **1**, obtained in 70% yield, was treated with dehydrating agents to give 2-methoxyxanthone **2**. 2-Hydroxyxanthone **3** was prepared by refluxing of **2** in 2,6-lutidine in the presence of LiI for 2 days or by heating of **2** in pyridine hydrochloride at 210° C for 3 h. The phenolic function of **3** was alkylated with ethyl 5-bromovalerate to give **4**. The free acid **5** could be collected after saponification of **4**. The 9-oxo group of **5** was selectively transformed to the corresponding amino function by reducing **5** with $NaBH_4$ followed by stirring the mixture with $(NH_4)HCO_3$ for 24 h at room temperature as reported by Han and associates [13]. 5-[[9-[(9-Fluorenylmethyloxycarbonyl)amino]xanthen-2-yl]oxy]-valeric acid (Fmoc-XAL_2 **7**) was prepared according to the standard literature method [18] and at-

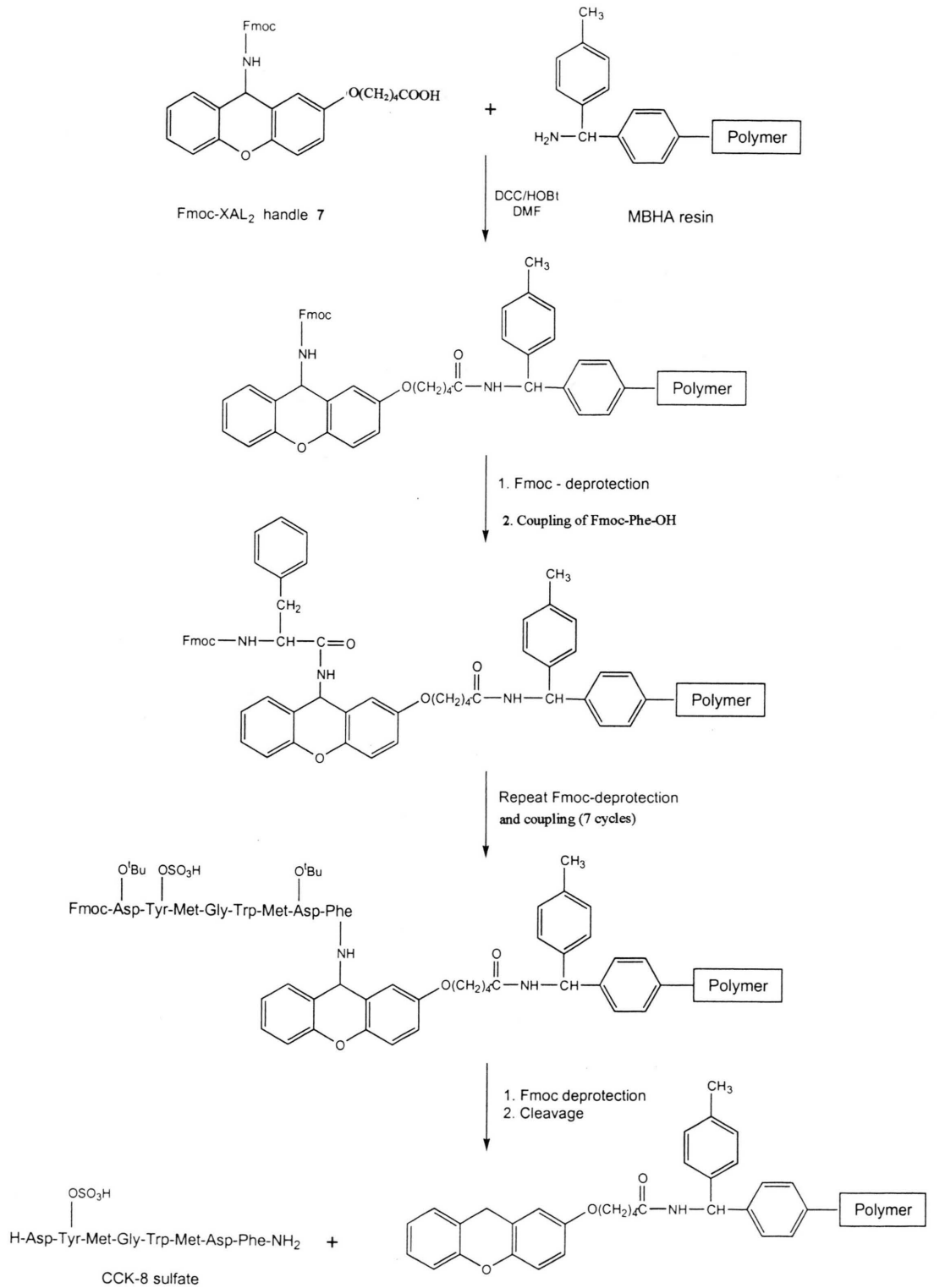
0932–0776/2001/0600–0526 $06.00



Scheme 1. Synthesis of Fmoc-XAL$_2$ handle **7**.

tached to 4-methylbenzhydrylamino-poly(styrene-co-1% divinylbenzene) resin. The coupling was allowed to proceed in a manual Merrifield vessel by shaking with DCC/HOBt in DMF at room temperature and gave 0.50–0.59 mmol/g loading.

Cholecystokinin sulfate ester [CCK-8 sulfate, Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2], an acid-labile octapeptide, which stimulates pancreatic exocrine secretion, gallbladder contraction, and may also act as a neurotransmitter in the central nervous system, was readily assembled by the stepwise Fmoc/^{t}Bu solid-phase procedure [19]. The side chain carboxyls of aspartates were protected as the *tert*-butyl esters, and other residues were unprotected. Fmoc-Tyr(SO_3Na)-OH was produced and tested in our laboratory. Coupling



Scheme 2. Synthesis of CCK-8 sulfate on XAL_2 handle.

of Fmoc amino acids to the Fmoc-XAL_2-MBHA polymer was performed using the DCC/HOBt protocol, except for Fmoc-Tyr(SO_3Na)-OH which was introduced *via* the BOP/HOBt/NMM method [20]. Upon completion of chain assembly, the peptidyl-resin was obtained in *ca.* 96% yield. As we found, the peptidyl-aminoxanthene bond could be selectively cleaved with 50% TFA in CH_2Cl_2 in the presence of scavangers (thiocresol:anisole:DMS, 1:1:4) at room temperature (Scheme 2). Different cleavage times (5, 15, 30, and 60 min) were used to check the peptide composition of the cleavage mixture. HPLC analysis indicated that 15 min cleavage proved to be the optimal one, yielding 45% sulfated peptide and 15% of unsulfated CCK-8, though *tert*-butyl protection of the side chain of the aspartates was still present to the extent of ~40% during this period. Desulfation became prominent (74%) and only 14% of sulfate ester could be detected using 60 min cleavage (Fig. 1). It should be noticed that these findings are significantly different from those reported by *Barany*'s group [13] using 15 min cleavage: ~70% of the total peptide material was the desired CCK-8 sulfate, and the cleavage mixture contained also 10% of unsulfated CCK-8 and only 20% of CCK-8 sulfate retaining *tert*-butyl side chain protection.

Asp-Gly-Leu-Tyr(PO_3H_2)-Gln-Gly-Leu-NH_2, a phosphotyrosine-containing heptapeptide of the ζ-subunit of the T-cell receptor complex was synthesized by SPPS Fmoc-methodology using the Fmoc-XAL_2-MBHA polymer and a modified synthesis protocol. The side chain of aspartic acid was protected by O^{t}Bu, the phenolic hydroxyl of the tyrosine residue remained unprotected. Couplings were performed with DCC with the exception of Gln, which was incorporated as HOBt ester. After coupling of Tyr, the phenolic hydroxyl was phosphitylated using di-*tert*-butyl-*N,N*-diethyl phosphoramidite [21], then the phosphite was oxidized to phosphate using *tert*-butyl hydroperoxide. Elongation of the peptide chain was completed and the resulting crude phosphopeptide was detached from the resin by TFA/DCM/ anisole (62:30:8) at 0° C within 1,5 h. One more hour was needed for the deprotection of the phosphate ester group. The free peptide was precipitated with diethyl ether, dissolved in water and lyophilized (Fig. 2).

In conclusion, our synthetic route to the 3-substituted xanthene derivative can be successfully applied for the large-scale preparation of 5-[(9-aminoxanthen-2-yl)oxy]valeric acid. The solid-phase application of this handle has been demonstrated for the syntheses of the acid-labile sulfated

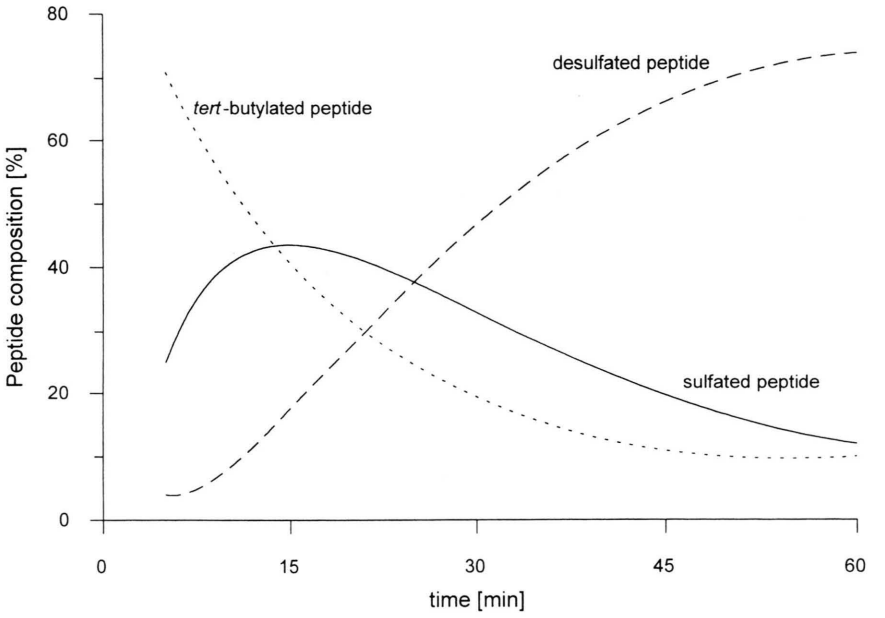


Fig. 1. Relationship between the crude peptide composition and cleavage time.

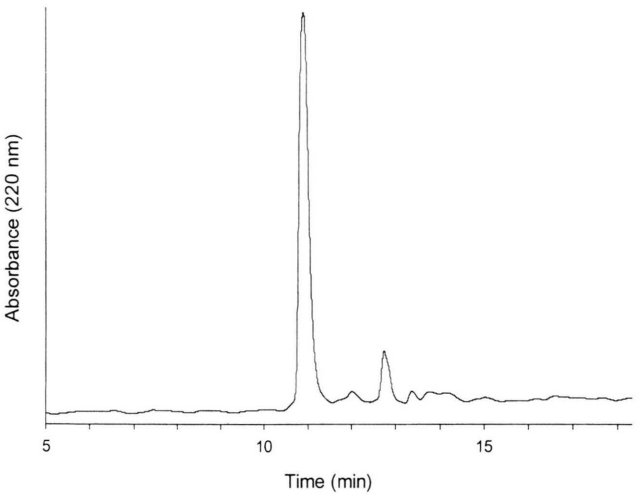


Fig. 2. HPLC profile of the crude phosphopeptide. Conditions: Lichrosorb-100 RP 18 Column (250×4.6 mm, 10 μm particle size); mobile phase 60% acetonitrile, 0.1% TFA; gradient elution from 5% to 80%; flow rate 0.8 ml/min.

and phosphorylated tryptophan-containing amino acid sequences.

Experimental

All reagents and solvents were of reagent grade and purchased from Sigma-Aldrich Kft (Budapest, Hungary). 9-Fluorenylmethylsuccinimidyl carbonate was received from Bachem (California, USA). Melting points were obtained with a PAMK VEB apparatus and are uncorrected. TLC was performed on silica gel precoated glass plates 60 F_{254} (Merck). Spectral data were acquired on a Bruker AN 400 MHz spectrometer (^{1}H NMR and ^{13}C NMR). Microanalyses were carried out with a CHN analyser (Prague).

2-[(4-Methoxyphenyl)oxy]benzoic acid (**1**)

o-Chlorobenzoic acid (50 g, 320 mmol) was dissolved in DMF (350 ml) and anhydrous K_2CO_3 (110 g, 596 mmol), 4-methoxyphenol (46 g, 362 mmol), Cu powder (1.28 g, 20 mmol), CuI (1.28 g, 6.74 mmol) and Clealand's reagent (1 g, 6.4 mmol) were added. The reaction mixture was heated under reflux for 6 h in N_2 atmosphere. DMF was evaporated in *vacuo*, then the oily residue was poured onto ice-water acidified with HCl to pH = 2. The crude product was precipitated as an oil, which solidified on standing overnight at 0° C. The brown precipitate was filtered, washed with water, dried and recrystallized from acetone/water, 54.8 g (70%). M.p. 139–141° C. – R_f = 0.26 (benzene:MeOH 9:1). – ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.82 (s, 3 H, OMe), 6.76–8.18 (8 H, aromatic H). – $C_{14}H_{12}O_4$ (242.2): calcd. C 68.85, H 4.95; found C 69.20, H 4.76.

2-Methoxyxanthone (**2**)

Acid **1** (60 g, 245.7 mmol) was dissolved in Ac_2O (400 ml) and concd H_2SO_4 (16 ml), the solution was stirred at 80° C for 1 h and poured onto ice-water. The greenish solid precipitate was filtered, washed with water to neutral, and dried. 50 g (90%) of product was obtained. An analytical sample was recrystallized from acetone/water. M.p. 132.5–134.5° C. – R_f = 0.63 (EtOAc/*n*-hexane 1:1). – ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.78 (s, 3 H, s, OMe), 7.13–8.22 (7 H, aromatic H). – $C_{14}H_{10}O_3$ (226.2): calcd. C 74.33, H 4.46; found C 74.67, H 4.33.

2-Hydroxyxanthone (**3**)

a.) 2-Methoxyxanthone (**2**, 32 g, 141.6 mmol) was dissolved in 2,6-lutidine (280 ml) and then anhydrous LiI (48 g, 358.8 mmol) was added. The mixture was heated under reflux and N_2 atmosphere for 2 days. After diluting with water, the solution was acidified with HCl to pH = 2. The precipitate was filtered, washed with water and dried to give **3** (29.2 g, 97%). An analytical sample was recrystallized from ethyl acetate/*n*-hexane. M.p. 238–242° C. – R_f = 0.42 (EtOAc/n-hexane 1:1).

b.) 2-Methoxyxanthone (**2**, 33 g, 146 mmol) and pyridine hydrochloride (100 g, 865 mmol) were

heated at 210° C for 3 h in N_2. The mixture was cooled and poured onto ice-water, acidified with HCl to pH = 2. The precipitate was filtered off, washed with water and dried, 29 g (94%). An analytical sample was recrystallized from acetone/*n*-hexane. M.p. 238–239° C. – R_f = 0.42 (EtOAc/*n*-hexane 1:1). – ^{1}H NMR (400 MHz, CD_3OD): δ = 3.30 (m, 1 H, OH), 7.28–8.24 (7 H, aromatic H). – $C_{13}H_8O_3$ (212.2): calcd. C 73.58, H 3.80; found C 73.73, H 3.94.

Ethyl 5-[(9-oxoxanthen-2-yl)oxy]valerate (**4**)

2-Hydroxyxanthone (**3**, 42 g, 198 mmol) was suspended in anhydrous acetone (2 l), then anhydrous K_2CO_3 (142 g, 1030 mmol) and ethyl 5-bromovalerate (81.9 ml, 514.5 mmol) were added. The reaction mixture was refluxed for 15 h with stirring. The cooled mixture was filtered to remove inorganic salts and washed with acetone. The filtrate was evaporated in *vacuo* to dryness and the residue was crystallized from EtOAc/*n*-hexane to give **4** (58.6 g, 87%) product. M.p. 62–64° C. – R_f = 0.67 (EtOAc/*n*-hexane 1:1). – ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.25 (b, J = 7.1 Hz, 3 H, Me), 1.83 (m, 4 H, CH_2), 2.38 (t, J = 6.9 Hz, 2 H, CH_2), 4.05 (m, 2 H, CH_2), 4.15 (q, J = 7.1 Hz, 2 H, CH_2), 7.24–8.32 (7 H, aromatic H). – $C_{20}H_{20}O_5$ (340.3): calcd. C 70.58, H 5.92; found C 70.85, H 6.13.

5-[(9-Oxoxanthen-2-yl)oxy]valeric acid (**5**)

Aqueous KOH (40 g, dissolved in 200 ml of water) was added to the suspension of ethyl ester **4** (82 g, 241.2 mmol) in MeOH (600 ml). After stirring for 1 h at 35–40° C, the reaction mixture was homogeneous and was poured onto ice-water, acidified with HCl to pH = 2. A white precipitate was formed quickly, which was collected, washed with water, and dried. Yield: 73.7 g (98%). M.p. 146–149° C. – R_f = 0.69 (CH_2Cl_2/MeOH 7:3). – ^{1}H NMR (400 MHz, CD_3SOCD_3): δ = 1.60–1.85 (m, 4 H, CH_2), 2.15 (t, J = 2 Hz, 2 H, CH_2), 4.10 (t, J = 2.1 Hz, 2 H, CH_2), 7.40–8.30 (7 H, aromatic H). – $C_{18}H_{16}O_5$ (312.3): calcd. C 69.22, H 5.16; found C 68.87, H 5.05.

5-[(9-Aminoxanthen-2-yl)oxy]valeric acid (**6**)

Keto acid **5** (30 g, 96 mmol) was suspended in H_2O, then dissolved by adding a solution of 1 M aqueous NaOH (110 ml, 110 mmol). $NaBH_4$ was added in a single portion. The reaction mixture was stirred for 2.5 h at 50–52° C, cooled to 10° C and solid $NaHCO_3$ (54 g, 200 equiv.) was added in small portions over 30 min under stirring, which was continued for 1 h at room temperature (checking by TLC). After adding $(NH_4)HCO_3$ (228 g, 450 mmol), stirring was continued for 24 h at room temperature while a white-yellow suspension was formed, which was acidified to pH = 5–6 by careful addition of 10% aqueous citric acid. The product was collected by filtration, washed with water, dried and used without further purification. Yield 27 g (90%), M.p. 133–143° C. – R_f = 0.25 (CH_2Cl_2/MeOH 7:3).

5-[[9-[(9-Fluorenylmethyloxycarbonyl)-amino]xanthen-2-yl]oxy]valeric acid (**7**)

$NaHCO_3$ (12.1 g, 144.4 mmol) was suspended in dioxane/water (1200 ml, *v/v* 1:1), then 5-[(9-aminoxanthen-2-yl)oxy]valeric acid **6** (22.6 g, 72.2 mmol) and Fmoc-ONSu (26.8 g, 79.5 mmol) were added. The reaction mixture was stirred for 10 h at room temperature, acidified with AcOH to pH = 6.3 while the product was precipitated as a light-yellow solid. The product was collected by filtration, washed with water, dried and crystallized from EtOAc/*n*-hexane. Yield 27.3 g (71%). M.p. 205–206° C. – R_f = 0.53 (EtOAc/MeOH 5:1). – ^{1}H NMR (400 MHz, CD_3SOCD_3): δ = 1.60–1.80 (m, 4 H, CH_2), 2.25 (t, J = 2 Hz, 2 H, CH_2), 3.90 (m, 2 H, CH_2), 4.27 (t, J = 5.8 Hz, 1 H, CH), 4.44 (d, J = 5.8 Hz, 2 H, CH_2), 5.92 (d, J = 7.6 Hz, 1 H, CH), 6.85–7.95 (15 H, aromatic H), 8.31 (d, J = 7.8 Hz, 1 H, NH). – $C_{33}H_{29}NO_6$ (535.60): calcd. C 74.00, H 5.45, N 2.61; found C 74.13, H 5.30, N 2.41.

Introduction of compound **7** *onto MBHA resin*

MBHA hydrochloride resin (70 g, 1.03 mmol/g) was swelled in CH_2Cl_2 (700 ml), then neutralized by 10% TEA/CH_2Cl_2. After washing by CH_2Cl_2, MeOH and CH_2Cl_2 (3 × with each), the coupling was done in DMF (700 ml) using Fmoc-XAL_2 (70 g, 130.7 mmol), DCC (26.9 g, 130.7 mmol) and HOBt (8.8 g, 65.4 mmol) for 24 h at room temperature (checking by Kaiser test [22]). The resin was filtered, washed with DMF, CH_2Cl_2 and MeOH, then dried. 120 g (0.50–0.59 mmol/g) of modified resin was obtained.

Analysis of the composition of the cleavage mixture for CCK-8 sulfate

After deprotection of the Fmoc group by 20% piperidine/DMF, the peptidyl resin (50 mg) was suspended in the cleavage coctail (TFA:CH_2Cl_2:-

thiocresol: anisole:DMS, 50:38:2:2:8, 1 ml) and stirred at room temperature for 5 min. The resin was filtered, washed with TFA (0.5 ml), and diisopropyl ether (5 ml) was added to the filtrate. The precipitate was collected by filtration and checked by HPLC. HPLC measurements were performed on a Hewlett Packard 1050 instrument [conditions: Lichrosorb 100 10RP 18 column (250×4.6 mm); mobile phase 60% acetonitrile, 0.1% TFA; gradient elution from 5% to 80%; flow rate 0.8 ml/min; 220 nm]. The intensities of the peaks was used for constructing Fig. 1. The same procedure was applied using cleavage times of 10, 15, 30, and 60 min.

Acknowledgements

The research was supported by the Hungarian Research Foundation (OTKA, No. 22551/1997). The authors wish to thank É. Menyhárth, É. M. Dósai and I. Nógrádi for the excellent technical assistance and R. Ferenci for his contribution preparing the manuscript.

[1] H. Rink, Tetrahedron Lett. **28,** 3787 (1987).
[2] G. Breipohl, J. Knolle, W. Stüber, Tetrahedron Lett. **28,** 5651 (1987).
[3] F. Albericio, G. Barany, Int. J. Peptide and Protein Res. **30,** 206 (1987)..
[4] B. Penke, J. Rivier, J. Org. Chem. **52,** 1197 (1987).
[5] S. Funakoshi, E. Murayama, L. Guo, N. Fujii, H. Yajima, J. Chem. Soc., Chem. Commun. **1988,** 382.
[6] B. Penke, L. Nyerges, N. Klenk, A. Asztalos, in B. Penke, A. Török (eds): The Peptides, p. 121, Walter de Gruyter, Berlin (1988).
[7] B. Penke, L. Nyerges, in G. Jung, E. Bayer (eds): The Peptides, p. 142, Walter de Gruyter, Berlin (1989).
[8] F. Albericio, N. Kneib-Cordonier, S. Biancalana, L. Gera, R. I. Masada, D. Hudson D, G. Barany, J. Org. Chem. **55,** 3730 (1990).
[9] J. Shao, Y.-H Li, W. Voelter, Int. J. Peptide Protein Res. **36,** 182 (1990).
[10] R. J. Bontems, P. Hegyes, S. L. Bontems, F. Albericio, G. Barany, in J. A. Smith, J. Rivier (eds): The Peptides, p. 601, ESCOM Science Publishers. B. V., Leiden (1992).
[11] P. Sieber, Tetrahedron Lett. **28,** 2107 (1987).
[12] W. C. Chan, S. L. Mellor, J. Chem. Soc., Chem. Commun. **1995,** 1475.
[13] Y. Han, L. S. Bontems, P. Hegyes, C. M. Munson, A. C. Minor, A. S. Kates, F. Albericio, G. Barany, J. Org. Chem. **61,** 6326 (1996).
[14] Cs. Somlai, P. Hegyes, L. Nyerges, B. Penke, W. Voelter, J. Prakt. Chem./Chem. Ztg **336,** 429 (1994).
[15] B. Penke, L. Nyerges, A. T. Szabó, M. Zarándi, in A. J. Smith, J. Rivier (eds): The Peptides, p. 617, ESCOM Science Publishers. B. V. Leiden (1992).
[16] B. Penke, L. Nyerges, A. T. Szabó, M. Zarándi, Proc. of the Symposium "Innovation and Perspectives in Solid-Phase Synthesis-1992", R. Epton (ed.): Collected Papers, p. 83, Intercept Limited: Andover (1992).
[17] F. Ullmann, M. Zlokastoff, Chem. Ber. **38,** 211 (1905) .
[18] L. Lapatsanis, G. Milias, K. Froussios, M. Kolovos, Synthesis **1983,** 671.
[19] G. B. Fields, R. L. Noble, Int. J. Peptide Protein Res. **35,** 161 (1990).
[20] B. Castro, J.-R. Dormoy, G. Evin, C. Selve, Tetrahedron Lett. **16,** 1219 (1978).
[21] I. Laczkó, M. Hollósi, E. Vass, Z. Hegedűs, É. Monostori, G. K. Tóth, Biophys. Biochem. Res. Commun. **242,** 474 (1998).
[22] E. Kaiser, R. L. Colescott, C. D. Bossiguer, P. I. Cook, Anal. Biochem. **34,** 595 (1970).