Active Esters of Formic Acid as Useful Formylating Agents: Improvements in the Synthesis of Formyl-Amino Acid Esters, *N*-α-Formyl-Met-Leu-Phe-OH, and Formyl-Met-Lys-Pro-Arg, a Phagocytosis Stimulating Peptide

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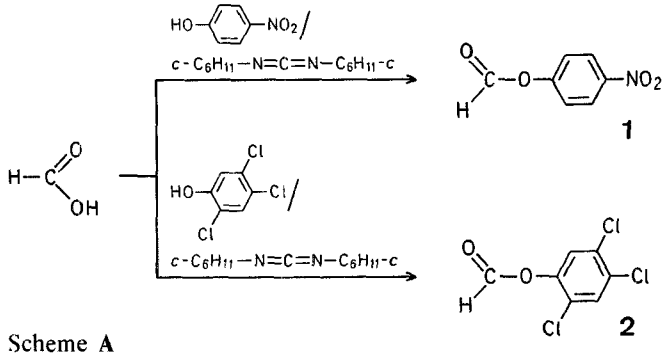
The formyl group is a useful amino-protecting group in peptide synthesis and can, for example, serve in combination with the selectively removable *t*-butoxycarbonyl group in the synthesis of suitable protected trifunctional amino acid derivatives. Another interesting aspect of formyl-amino acid esters is their ready conversion to isocyano acid esters which are required as one of the starting material in four component condensations[1].

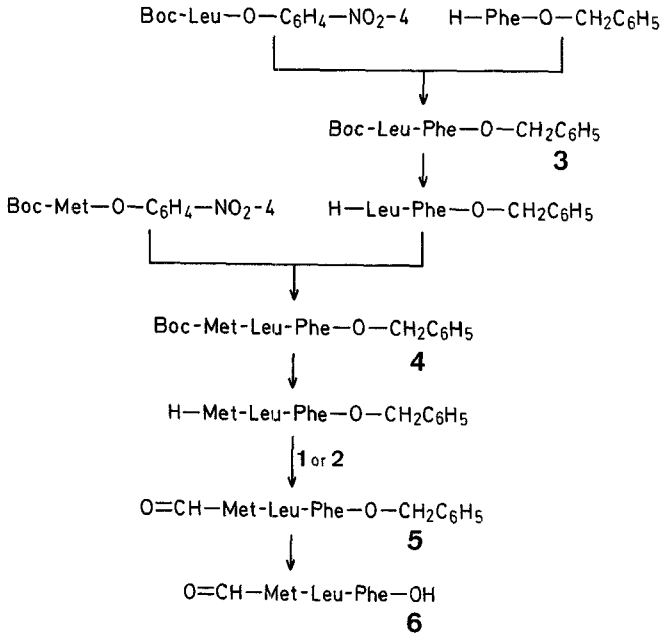
The formylation of amino groups is generally achieved by reaction of the amine with formic acid and acetic anhydride[2]. However, it has been found in many cases that this method is somewhat unreliable. Recently, other methods for preparing formyl-amino acids and formyl-peptides have been proposed: *t*-butyl esters of amino acids have been formylated using formic acid and dicyclohexylcarbodiimide[3], and the same system has been used in the synthesis of *N*-α-formyl-Met-Leu-Phe, a chemotactic peptide[4]. One of the main disadvantages of this method is the formation of dicyclohexylurea as side product which is very difficult to remove from the above chemotactic peptide. Recently, the use of 3-methylbutanoyl chloride or 2,2-dimethylpropanoyl chloride in the presence of formic acid has been proposed for the introduction of the formyl group[5]. Disadvantages such as odor and unpractical use of these acid chlorides prompted us to search for a further interesting, clean, and rapid method of formylation. One of the cleaner methods in peptide synthesis is the active ester method[6]. *p*-Nitrophenyl formate **(1)** and *o*-nitrophenyl formate have been prepared[7] but only rarely used in peptide synthesis.

We have now prepared *p*-nitrophenyl formate **(1)** and 2,4,5-trichlorophenyl formate **(2)** (Scheme **A**) and studied their use in the synthesis of formyl-amino acid esters and formyl-peptides.



Scheme **A**

The active formate esters **1** and **2** were prepared by reaction of formic acid with the corresponding phenol in the presence of dicyclohexylcarbodiimide, the products being obtained after 3 h by precipitation from the reaction mixture by ethanol/water. Compound **2** is the more stable and can be stored without any special precautions. Both esters **1** and **2** show high reactivity towards nucleophiles and were used to prepare formyl-Leu-$OCH_2C_6H_5$ and formyl-Ala-OC_4H_9-*t* in good yield and high purity. The usefulness of **1** or **2** was further demonstrated by the synthesis of the chemotactic peptide **6** (Scheme **B**). Each intermediate was obtained in a high degree of purity and high yield. The formyl group was introduced using either **1** or **2** and the peptide **5** was obtained as a pure, crystalline material in high yield.



Scheme **B**

The difficulties reported in preparing this peptide in pure form[4] were not encountered when using **1** or **2**. Side products obtained during the synthesis of formyl-amino acid *t*-butyl esters[3] were not detected.

The protected peptide **5** was hydrogenated in a mixture of dimethylformamide, diisopropylethylamine, and water in the presence of 10% palladium on barium sulfate[8] to give **6** as pure, colorless needles. The usual hydrofluoric acid treatment at this stage is advantageously replaced by hydrogenation. The chemotactic peptide **6** was identified by comparison of its physical properties with those given for an authentic sample prepared by other routes.

Ester **2** was also used to prepare the partially protected peptide **13** (Scheme **C**). The peptide Leu-Lys-Pro-Arg, an analog

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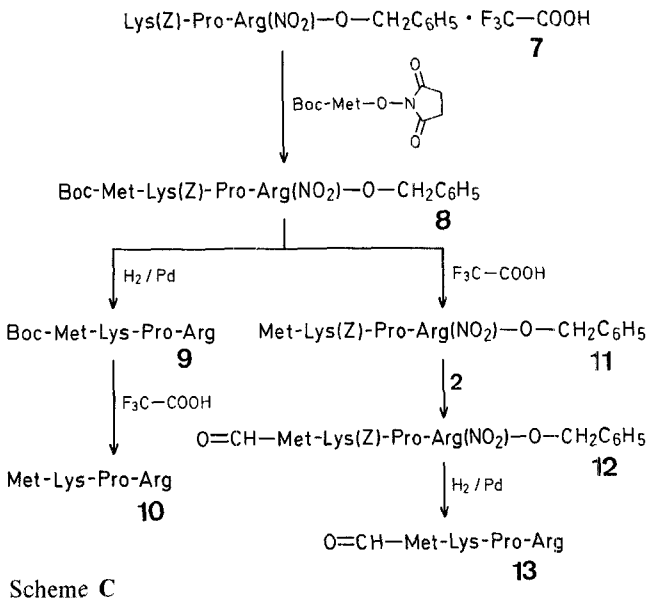
of Tuftsin[9], is highly potent in stimulating phagocytosis of polymorphonuclear leucocytes[10]. It is well known that methionine and leucine can replace each other in natural analogs of several biologically active peptides (e.g. porcine and human gastrin, enkephalins, etc.). We thus expected that replacement of leucine by methionine in this Tuftsin analog would result in an active product. However, it was recently reported that, in addition to promoting cell locomotion, a further biological function of leucoattractants (formyl-peptides) may be their capacity to render complement receptors more freely available, thereby increasing the magnitude of adhesion of phagocytic cells to opsonized particles[11]. Thus, **13** could be a possible leucoattractant and phagocytosis-stimulating peptide.



Scheme C

The partially protected tripeptide trifluoroacetate salt **7**[12] was reacted with the protected methionine[13], the resultant fully protected tetrapeptide **8** was hydrogenated as described above to give **9** which was purified by silica gel chromatography. Treatment of **9** with trifluoroacetic acid gave, after purification, Met-Lys-Pro-Arg **(10).** Alternatively, treatment of the peptide **8** with trifluoroacetic acid gave the trifluoroacetate salt of the peptide **11.** Reaction of **11** with reagent **2** gave the formyl-peptide **12** which was partially deprotected by hydrogenation as described previously to give formyl-Met-Lys-Pro-Arg **(13).** Both peptides **10** and **13** are able to stimulate phagocytosis of polymorphonuclear leucocytes, the results of these studies will be published elsewhere.

The main advantages of using the active esters **1** or **2** are:
- the ester **2** can be simply prepared and stored,
- formyl-amino acids and formyl-peptides are obtained in high yield and high purity,
- the process is experimentally simple.

Capillary melting points were determined in a Tottoli apparatus and are uncorrected. Thin layer chromatograms were run on commercial silica gel plates (Merck). Spots were detected by U.V. absorption and by charring. Elemental analyses were performed by "Le Service Central de microanalyse du CNRS de Montpellier". I.R. spectra were determined with a Beckmann Acculab 4 spectrophotometer in KBr pellets, ^{1}H-N.M.R. spectra for 10% solutions in $CDCl_3$ with a Varian A60 spectrophotometer using TMS as internal standard (chemical shifts are reported in δ values of ppm units). Amino acids of L-configuration are used.

p-Nitrophenyl Formate (1):
To a cooled (0 °C) solution of 98% formic acid (3 g) in ethyl acetate (50 ml) are added *p*-nitrophenol (7.6 g, 5.5 mmol) and dicyclohexylcarbodiimide (11.3 g, 5.5 mmol). The mixture is stirred at 0 °C for 1 h and at room temperature for 3 h. The precipitated dicyclohexylurea is filtered and washed with ethyl acetate (2 × 50 ml). The combined organic solution is washed with saturated sodium hydrogen carbonate solution (1 × 50 ml), water (2 × 50 ml), dried with sodium sulfate, and concentrated in vacuum at 40 °C. The residue is dissolved in ethanol (50 ml), addition of water (150 ml) gives a crystalline precipitate which is rapidly filtered, washed with cold ethanol (2 × 10 ml), and dried in vacuo. This product is not very stable when not absolutely pure; yield: 6 g (72%); m.p. 65–68 °C.

$C_7H_5NO_4$	calc.	C 50.31	H 3.02	N 8.38
(167.1)	found	50.44	3.05	8.37

2,4,5-Trichlorophenyl Formate (2):
Similarly prepared from 2,4,5-trichlorophenol; it can be stored at room temperature; yield: 75%; m.p. 68–70 °C (Ref.[14], m.p. 66–67 °C). It was recrystallized brom hexane.

$C_7H_3Cl_3O_2$	calc.	C 37.28	H 1.34	Cl 47.16
(225.5)	found	37.59	1.53	46.98

I.R. (KBr): $\nu = 1730$ cm^{-1} (C=O).

Formyl-L-Leucine Methyl Ester:
A solution of Leu-OCH_3 hydrochloride (0.80 g, 4.4 mmol) in dimethylformamide (5 ml) containing diisopropylethylamine (0.76 ml, 4.4 mmol) is cooled in an ice bath. 2,4,5-Trichlorophenyl formate (**2**; 0.902 g, 4.0 mmol) is added and, after 30 min at 0 °C, the reaction mixtures is allowed to warm to room temperature over 2 h. The solution is concentrated in vacuo and the residue is dissolved in ethyl acetate (50 ml). The organic solution is washed with saturated sodium hydrogen carbonate solution (2 × 25 ml), water (25 ml), 5% citric acid solution (25 ml), water (25 ml), dried with sodium sulfate, and concentrated in vacuo. Filtration through a silica gel column gives a colorless oil which is homogeneous by T.L.C. and is identified by its ^{1}H-N.M.R. spectrum; yield: 75%.

^{1}H-N.M.R. ($CDCl_3$): $\delta = 0.95$ (d, 6H, CH_3); 1.70 (3H, —CH—CH_2—); 3.75 (s, 3H, OCH_3); 4.75 (m, 1H, CH—CO—); 7.30 (d, 1H, NH); 8.25 ppm (s, 1H, —CH=O).

Formyl-L-alanine t-butyl ester is prepared similarly; yield: 78%.

^{1}H-N.M.R. ($CDCl_3$); $\delta = 1.4$ (d, 3H, CH_3); 1.5 (s, 9H, *t*-C_4H_9); 4.1 (m, 1H, CH—CO—); 7.30 (d, 1H, NH); 8.25 ppm (s, 1H, —CH=O).

Boc-Leu-Phe-$OCH_2C_6H_5$ (3):
To a cooled (0 °C) solution of Boc-L-Leu-O-succinimide (Bachem; 2.5 g, 7.9 mmol) in dimethylformamide (10 ml) is added the *p*-toluenesulfonate salt of L-Phe-$OCH_2C_6H_5$ (3.71 g, 8.7 mmol) and diisopropylethylamine (1.3 ml) in dimethylformamide (10 ml). The mixture is stirred at room temperature for 12 h and then concentrated in vacuo at 40 °C. The residue is dissolved in ethyl acetate (150 ml), the organic layer is washed as described above, dried with sodium sulfate, and concentrated in vacuo; yield: 2.8 g (75%); m.p. 103–105 °C (from ethyl acetate/light petroleum ether); $[\alpha]_D^{20}$: −20.9° (*c* 1.1, DMF); homogeneous by T.L.C.

$C_{27}H_{36}N_2O_5$	calc.	C 69.18	H 7.76	N 5.98
(468.7)	found	69.04	7.68	5.80

Boc-Met-Leu-Phe-$OCH_2C_6H_5$ (4):
Compound **3** (2.7 g, 5.8 mmol) is treated with trifluoroacetic acid (10 ml) for 40 min. The trifluoroacetic acid is evaporated in vacuo and the residue is triturated with ether (150 ml). The crystalline precipitate is filtered, the solid is rinsed with ether, dissolved in dimethylformamide (10 ml), and cooled at 0 °C. Diisopropylethylamine (3 ml, 14.2 mmol) is added, followed by Boc-Met-OC_6H_4-NO_2-4 (2.40 g, 6.5 mmol) and 1-hydroxybenzotriazole (0.99 g, 6.5 mmol) in dimethylformamide (10 ml). After 12 h at room temperature the solvent is evaporated in vacuo, the residue is dissolved in ethyl acetate (200 ml) and treated as described for **3.** The resultant crystalline material is washed many times with ether and collected by filtration; yield: 2.8 g (76%); m.p. 142–144 °C; $[\alpha]_D^{20}$: −32.0° (*c* 1.8, DMF); homogeneous by T.L.C.

$C_{32}H_{45}N_3O_6S$	calc.	C 64.06	H 7.58	N 7.01
(599.9)	found	63.85	7.48	6.76

Formyl-Met-Leu-Phe-$OCH_2C_6H_5$ (5):
Compound **4** (1.2 g, 2 mmol) is treated with trifluoroacetic acid (10 ml) for 40 min. The mixture is then evaporated in vacuo, the residue is treated with ether (100 ml), filtered, and the solid washed several times with ether. This product (0.5 g, 0.94 mmol) is dissolved in dimethylformamide (3 ml) and cooled to 0 °C. Diisopropylethylamine (0.2 ml) and 2,4,5-trichlorophenyl formate (**2**; 0.27 g, 1.2 mmol) are added. After 12 h at room temperature, the mixture is treated as described above. The crystalline residue is triturated with ether, filtered, and washed several times with ether; yield: 0.387 g (92%); m.p. 134–136 °C; $[\alpha]_D^{20}$: −24.1° (*c* 1.0, DMF).

$C_{28}H_{37}N_3O_5S$	calc.	C 63.71	H 7.08	N 7.96
(527.8)	found	63.66	6.88	7.78

Formyl-Met-Leu-Phe (6):
Compound **5** (0.25 g) is hydrogenated in 10:1:1 dimethylformamide/diisopropylethylamine/water (20 ml) with 10% palladium on barium sulfate as catalyst (30 mg) for 24 h. Most of the catalyst is then removed by filtration and the solution is concentrated in vacuo. The residue is dissolved in dimethylformamide (20 ml) and filtered through a column of neutral alumina (5 g). Elution is continued with dimethylformamide. Concentration of the solution and trituration of the residue with ether containing 1% acetic acid gives a white powder; yield: 0.22 g (80%); m.p. 220 °C; $[\alpha]_D^{20}$: −10.0° (*c* 1.04, acetic acid); homogeneous by T.L.C.

$C_{21}H_{31}N_3O_5S$	calc.	C 57.62	H 7.15	N 9.60
(437.7)	found	57.48	7.09	9.58

Boc-Met-Lys(Z)-Pro-Arg(NO_2)-$OCH_2C_6H_5$ (8):
To a solution of the bis-trifluoroacetate salt of Lys(Z)-Pro-Arg(NO_2)-$OCH_2C_6H_5$[12] (**7**; 1.8 g, 2.0 mmol) in dimethylformamide (5 ml) containing diisopropylethylamine (0.72 ml, 4.2 mmol) is added Boc-Met-OC_6H_4-NO_2-4[16] (0.735 g, 2.2 mmol). The mixture is stirred for 12 h and then concentrated in vacuo. The oily residue is dissolved in ethyl acetate (50 ml), washed as described above, dried with magnesium sulfate, and concentrated in vacuo. Trituration of the residue with ether yields the pure product; yield: 1.5 g (85%); m.p. 80–85 °C; $[\alpha]_D^{20}$ −32.0° (*c* 1, DMF); R_F: 0.3 (95:5 acetic acid/methanol); R_F: 0.8 (80:20:5:8 ethyl acetate/pyridine/acetic acid/water).

$C_{42}H_{61}N_9O_{11}S$	calc.	C 56.03	H 6.84	N 14.00
(900.3)	found	56.20	6.68	13.68

Met-Lys-Pro-Arg (10):
Compound **8** (0.456 g, 0.5 mmol) is dissolved in 10:1:1 dimethylformamide/diisopropylethylamine/water (20 ml) containing 10% palladium on barium sulfate (50 mg) and hydrogenated for 48 h. Most of the catalyst is removed by filtration and the solution is concentrated in vacuo at 40 °C. The resultant oily residue is dissolved in dimethylformamide (20 ml) and filtered through a column of neutral alumina (30 g). Elution is performed with dimethylformamide. The solution is then concentrated in vacuo and the residue is treated with trifluoroacetic acid (15 ml) containing *N*-acetylmethionine *n*-butyl ester[15] (1 g) for 40 min. Addition of ether (150 ml) gives a white, hygroscopic precipitate which is purified by chromatography on silica gel using 20:20:5:15 ethyl acetate/pyridine/acetic acid/water as eluent; yield: 0.142 g (54%); R_F: 0.4 (20:20:5:15 pyridine/acetic acid/water/ethyl acetate); R_F: 0.1 (4:1:1 *n*-butanol/acetic acid/water).

Amino acid analysis: Met, 1.02; Lys, 1.01; Pro, 1.00; Arg, 0.98.

Formyl-Met-Lys(Z)-Pro-Arg(NO_2)-$OCH_2C_6H_5$ (12):
Compound **8** (0.456 g, 0.5 mmol) is treated with trifluoroacetic acid (10 ml) containing *N*-acetylmethionine *n*-butyl ester[15] (0.5 g) for 40 min. Ether (150 ml) is then added, the white precipitate collected by centrifugation, rinsed with ether (3 × 30 ml), dissolved in dimethylformamide (5 ml), and cooled to 0 °C. 2,4,5-Trichlorophenyl formate (**2**; 0.135 g, 0.6 mmol) is added and, after 12 h at room temperature, the solvent is removed in vacuo. The residue is dissolved in ethyl acetate (100 ml), the organic solution is washed with saturated sodium hydrogen carbonate solution (30 ml), water (2 × 30 ml), dried with magnesium sulfate, and concentrated in vacuo. The oily residue is purified by chromatography on silica gel using ethyl acetate as eluent; yield: 0.236 g (55%); m.p. 75–80 °C; $[\alpha]_D^{20}$: −30.4° (*c* 2.3, DMF); R_F: 0.4 (95:5 ethyl acetate/methanol).

$C_{38}H_{53}N_9O_{10}S$	calc.	C 55.11	H 6.46	N 15.23
(828.1)	found	55.03	6.30	15.44

Formyl-Met-Lys-Pro-Arg (13):
Compound **12** (0.17 g, 0.2 mmol) is hydrogenated as described for compound **8** purified similarly, and lyophilized in 3% aqueous acetic acid; yield: 0.068 g; m.p. 180 °C; R_F: 0.5 (20:20:5:15 ethyl acetate/pyridine/acetic acid/water); R_F: 0.2 (4:1:1 *n*-butanol/acetic acid/water); $[\alpha]_D^{20}$: −30.3° (*c* 1.21, 1:1 DMF/1% hydrochloric acid).

Amino acid analysis: Met, 0.98; Lys, 0.97; Pro, 1.01; Arg, 0.98.

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