# Use of Dimethylformamide–Sulphur Trioxide Complex as a Sulphating Agent of Tyrosine

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Dimethylformamide–sulphur trioxide (DMF–$SO_3$) complex was found to be more suitable for tyrosine sulphation than pyridine–sulphur trioxide (Pyr–$SO_3$) complex, the most commonly used sulphur trioxide complex for sulphation. The work-up after sulphation using DMF–$SO_3$ was also easier than with Pyr–$SO_3$. The usefulness of DMF–$SO_3$ complex was demonstrated through the synthesis of two tyrosine sulphate [Tyr($SO_3H$)]-containing peptides, leucine-enkephalin sulphate and leucosulfakinin-II.

Recently various Tyr($SO_3H$)-containing peptides and proteins have been found from various species[1] and tyrosine sulphation has become to be regarded as one of the most general post-translational modifications.[2]† However, the essential role of tyrosine sulphation towards the organisms is still uncertain. Synthetic Tyr($SO_3H$)-containing peptides would provide useful information in this respect.

From a synthetic point of view, the synthesis of Tyr($SO_3H$)-containing peptides is still a challenge. Among the crucial problems is the selection of a sulphating agent for tyrosine residue in peptides. Though concentrated sulphuric acid[3] is sometimes used, it can give 3′-sulphonyl-tyrosine as a major side product. Penke *et al.*[4] introduced pyridinium acetyl sulphate (PAS) as a sulphating agent of tyrosine and the sulphation in the synthesis of porcine cholecystokinin (CCK)-33[1d] was achieved in TFA by this agent.[5] However, when unprotected tryptophans are present in the substrate for sulphation, damage of the indole ring caused by treatment with PAS in TFA would be inevitable. In such cases, it would be preferable to sulphate tyrosine under neutral or mildly basic conditions. Fujii *et al.*[6] reported that the Pyr–$SO_3$ complex was more suitable than PAS for tyrosine sulphation in DMF–pyridine. However, when sulphation is conducted with the Pyr–$SO_3$ complex, the reaction media tend to be coloured brown. Moreover the work-up after sulphation is sometimes not easy because Pyr–$SO_3$ complex is not readily soluble to water, ether, *etc.*

Here we report the use of the DMF–$SO_3$ complex[7] as a useful sulphating agent of Tyr. Since DMF is a weaker base than pyridine, the partial positive charge on the sulphur atom of the DMF–$SO_3$ complex would be greater than that in the Pyr–$SO_3$ complex. The sulphation ability of the DMF–$SO_3$ complex would thus be expected to be greater than that of the Pyr–$SO_3$ complex.

First, to test this hypothesis, we conducted a model experiment. Boc-Tyr-OH was sulphated with DMF–$SO_3$ or Pyr–$SO_3$ complex (5 equiv. each) in DMF in the presence of pyridine at 25 °C. The progress of the reaction was followed by high performance liquid chromatography (HPLC). As expected, the rate of sulphation by DMF–$SO_3$ was about 20% greater than that with the Pyr–$SO_3$ complex (Figure 1).

To examine practical utility of this agent, we synthesized two model peptides: leucine-enkephalin sulphate[1a] and leucosulfakinin (LSK)-II.[1c] Leu-enkephalin sulphate was found by Unsworth *et al.* in 1982 from mouse striatum. They reported that sulphation on Tyr caused significant diminution of naloxon-reversible activity.[1a]

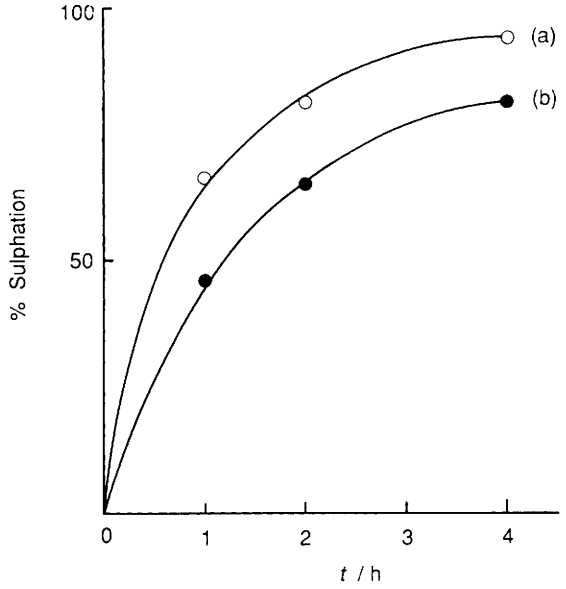
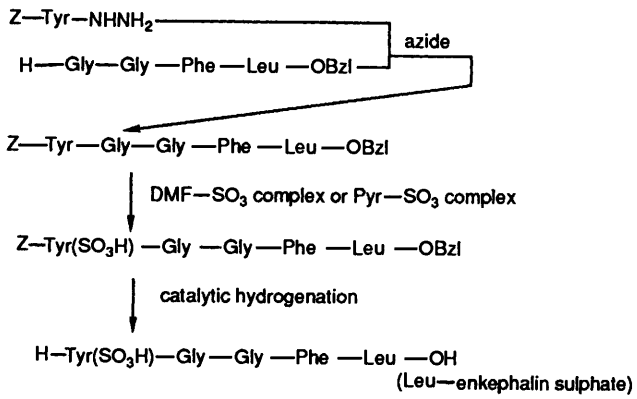



**Figure 1.** Sulphation of Boc-Tyr-OH by (*a*) DMF–$SO_3$ or (*b*) Pyr–$SO_3$ complex.

The procedure for the synthesis of Leu-enkephalin sulphate is shown in Scheme 1. Since Leu-enkephalin does not contain Met residues, final deprotection was conducted by catalytic hydrogenation. Z-Tyr-Gly-Gly-Phe-Leu-OBzl was prepared by the azide condensation of Z-Tyr-$NHNH_2$ and a TFA-treated sample of Z(OMe)-Gly-Gly-Phe-Leu-OBzl,[8] then subjected to sulphation with DMF–$SO_3$ complex (20 equiv.) in DMF–pyridine at 30 °C for 2 h. Since DMF–$SO_3$ complex is readily soluble in water and decomposes to DMF and $H_2SO_4$, the excess of agent was easily separated from the desired product.

† Amino acids used in this paper are of the L-configuration. The following abbreviations are used: Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = t-butoxycarbonyl, Z = benzyloxycarbonyl, Bzl = benzyl, $Cl_2Bzl$ = 2,6-dichlorobenzyl, Chp = cycloheptyl, Mts = mesitylenesulphonyl, (o) = sulphoxide, Su = *N*-hydroxysuccinimidyl, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulphonic acid, MSA = methanesulphonic acid, EDT = ethane-1,2-dithiol, NMM = *N*-methylmorpholine, TEA = triethylamine, CHA = cyclohexylamine, DMF = *N*,*N*-dimethylformamide, LAP = leucine aminopeptidase, Pyr = pyridine, pGlu = pyroglutamyl.




**Scheme 1.**

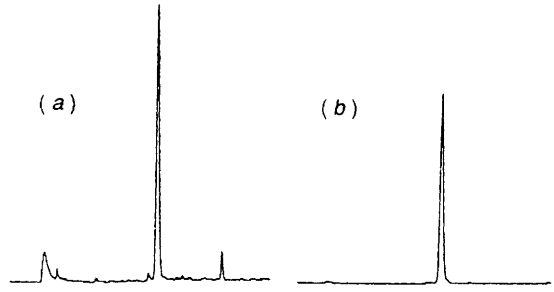



**Figure 2.** HPLC of the synthetic Leu-enkephalin sulphate: (*a*) gel-filtered sample; (*b*) HPLC-purified sample. Column: YMC AM-312 (6 × 150 mm). Elution: gradient elution with MeCN (15–35% in 20 min) in 0.1M $AcONH_4$ (pH 6.5) at a flow rate of 1 ml/min. Detection: 275 nm.

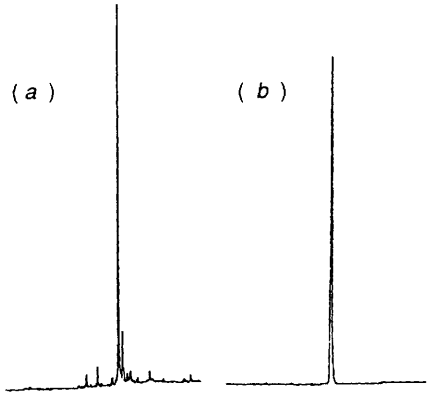



**Figure 3.** HPLC of the synthetic non-sulphated leucosulfakinin-II: (*a*) gel-filtered sample; (*b*) HPLC-purified sample. Column: YMC AM-312 (6 × 150 mm). Elution: gradient elution with MeCN (15–40% in 30 min) in 0.1% TFA at a flow rate of 1 ml/min. Detection: 275 nm.

Deprotection by catalytic hydrogenation with Pd/C and subsequent purification by HPLC afforded pure Leu-enkephalin sulphate in 66% yield (Figure 2). On the other hand, when the sulphation was conducted with Pyr–$SO_3$ complex for comparison, it took 4 h for complete sulphation. Moreover, since the Pyr–$SO_3$ complex is not readily soluble in water or ether, it was difficult to remove the excess completely, and the protected pentapeptide sulphate obtained was pale brown. The yield (55%) after the deprotection and subsequent purification was lower than that with the DMF–$SO_3$ complex.

To evaluate further the usefulness of the DMF–$SO_3$ complex, another Tyr($SO_3H$)-containing peptide, LSK-II, was synthesized. LSK-II is a neuropeptide isolated from head extracts of the cockroach, *Leucophaea maderae*, and has structural similarity

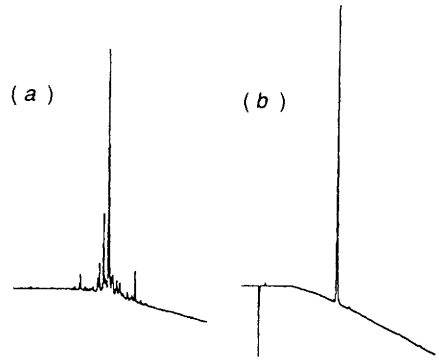


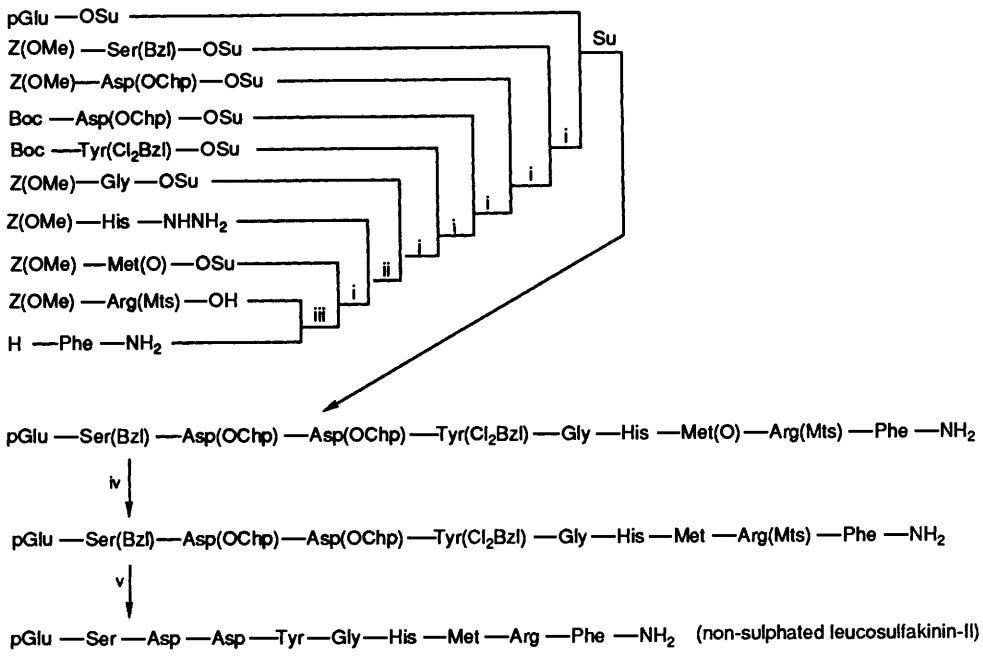

**Figure 4.** HPLC of the synthetic leucosulfakinin-II: (*a*) gel-filtered sample; (*b*) HPLC-purified sample. Column: YMC AM-312 (6 × 150 mm). Elution: gradient elution with MeCN (10–40% in 30 min) in 0.1M $AcONH_4$ (pH 6.5) at a flow rate of 1 ml/min. Detection: 215 nm.

with CCK and/or gastrin in mammals.[1c] Since LSK-II contains a Met residue, catalytic hydrogenation cannot be used for the final deprotection, which must be conducted under acidic conditions. In view of the acid-lability of Tyr($SO_3H$),[9] sulphation should be conducted after final acid-deprotection. Here, preferential protection of Ser prior to sulphation of Tyr becomes necessary, since sulphation occurs preferentially on the alcoholic hydroxy group of Ser rather than the phenolic hydroxy group of Tyr.[4,6] This problem was solved by preferential re-protection of the hydroxy group of Ser in the deprotected peptide with the $Bu^tPh_2Si$[10] group followed by sulphation of the hydroxy group of Tyr (Scheme 3), procedures which were developed by Fujii *et al.*[6] in the total synthesis of human CCK-33.[1e]

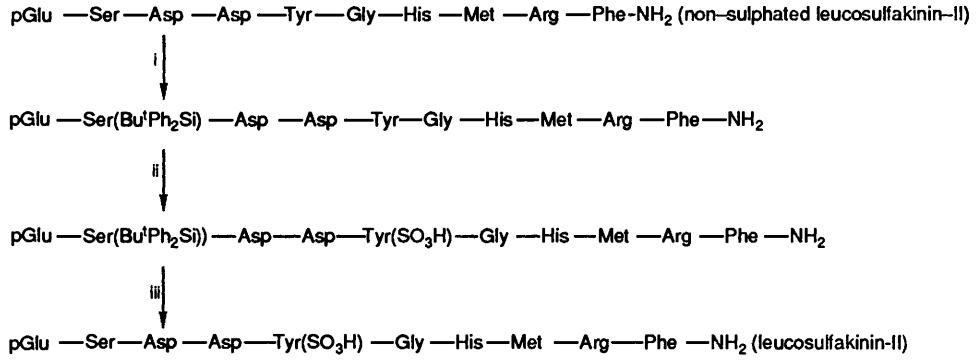
First, to prepare the non-sulphated LSK-II, the protected decapeptide amide was synthesized in a stepwise manner starting with H-Phe-$NH_2$ as shown in Scheme 2. For the protection of side-chain functional groups, Ser(Bzl), Asp(OChp),[11] Tyr($Cl_2$Bzl),[12] Met(O),[13] and Arg(Mts)[14] were employed. Peptide chain elongation was carried out mainly by the Su[15] active-ester procedure. The fully protected decapeptide amide was obtained without notable difficulties.

Prior to the final deprotection, the Met(O) residue of the protected peptide was reduced to Met with the DMF–$SO_3$ complex/EDT system[16] at 30 °C in 2 h. This novel reduction system was recently introduced by us and was expected to save the time required for the reduction of Met(O) and ensure the completion of the reduction. The reduced protected peptide was then treated with 1M TFMSA–thioanisole/TFA[17] to remove all the protecting groups. Subsequent purification by gel-filtration on Sephadex G-10 and HPLC on a YMC AM-323 column afforded non-sulphated LSK-II in 40% yield (Figure 3).

Sulphation of the foregoing unsulphated peptide was conducted as shown in Scheme 3. First, the hydroxy group of Ser was preferentially protected with the $Bu^tPh_2Si$ group by treatment of the unsulphated peptide with $Bu^tPh_2SiCl$ and imidazole in the presence of phenol. After gel-filtration on Sephadex LH-20, sulphation of Tyr was conducted with the DMF–$SO_3$ complex in DMF–pyridine at 30 °C for 36 h. The reaction medium was scarcely coloured by this agent. After gel-filtration on Sephadex LH-20, the $Bu^tPh_2Si$ group was removed by treatment with $Bu_4NF$. Subsequent purification by gel-filtration on Sephadex G-10 and HPLC on YMC-AM 312 afforded analytically pure LSK-II in 11% yield (Figure 4). The presence of Tyr($SO_3H$) was confirmed by Fourier transform infrared spectrometry (FTIR), amino acid analysis after $Ba(OH)_2$ hydrolysis,[18] and fast atom bombardment mass spectrometry (FABMS). When sulphation was conducted with the Pyr–$SO_3$ complex in the above experiment, separation of unchanged




**Scheme 2.** *Reagents:* i, Su, then TFA; ii, azide, then TFA; iii, MA, then TFA; iv, reduction of Met(O) by DMF–$SO_3$ complex/EDT; v, deprotection by 1M TFMSA–thioanisole/TFA.




**Scheme 3.** *Steps:* i, preferential masking of Ser by $Bu^tPh_2SiCl$; ii, sulphation of Tyr by DMF–$SO_3$ complex; iii, removal of $Bu^tPh_2Si$ group by $Bu_4NF$.

Pyr–$SO_3$ complex from the sulphated peptide was not easy with gel-filtration on Sephadex LH-20, and the total yield was 5%.

In conclusion, the use of the DMF–$SO_3$ complex was advantageous for the sulphation of Tyr compared with the conventionally used Pyr–$SO_3$ complex.

## Experimental

M.p.s were determined with a Yanagimoto micro-apparatus and are uncorrected. Optical rotations were determined with a Union PM–201 polarimeter. HPLC was conducted with a Hitachi L-6200 model. Amino acid compositions in acid hydrolysates (6M HCl in the presence of phenol; or 4M MSA,[19] 110 °C, 24 h), $Ba(OH)_2$ hydrolysates[18] [0.2M $Ba(OH)_2$, 110 °C, 20 h], and LAP digests[20] (Sigma, No. L-6007) were determined with a Hitachi 835 model amino acid analyser. FAB mass spectra were recorded on a JEOL JMS-D 300 spectrometer or a VG Analytical ZAB SE instrument. FT IR spectra were obtained on a Perkin-Elmer 1720 spectrometer. Silica gel 60 (Merck, 70–230 mesh) was used for column chromatography. $R_f$ values in TLC (pre-coated Silica gel 60 $F_{254}$, 0.25 mm thickness, Merck) refer to the following solvent systems: $R_{f1}$ $CHCl_3$–MeOH–$H_2O$ (8:3:1), $R_{f2}$ $CHCl_3$–MeOH (10:0.5), $R_{f3}$ $CHCl_3$–MeOH–AcOH (9:1:0.5), $R_{f4}$ $Bu^nOH$–AcOH–AcOEt–$H_2O$ (1:1:1:1), $R_{f5}$ $Bu^nOH$–AcOH–pyridine–$H_2O$ (4:1:1:2).

DMF–$SO_3$ complex was purchased from Fluka. Pyr–$SO_3$ complex was purchased from Tokyo Chemical Industry Co., Ltd. Authentic Leu-enkephalin sulphate was purchased from Peptide Institute, Inc., Osaka.

Prior to condensation, the $N^{\alpha}$-Z(OMe) or Boc group was removed by treatment with TFA in the presence of anisole. Active ester reactions were carried out at room temperature, and mixed anhydride (MA) reaction[21] in an ice bath. Azides were prepared according to the procedure of Honzl and Rudinger[22] and each reaction was carried out at 4 °C. Unless otherwise mentioned, products were purified by one of the following procedures. *Procedure A*: After evaporation of the solvents, the product was extracted with AcOEt. The extract was washed with 5% citric acid, 5% $NaHCO_3$, and NaCl–$H_2O$, then dried over $Na_2SO_4$ and concentrated. The residue was recrystallized or precipitated from appropriate solvents. *Procedure B:* After evaporation of the solvents, the residue was triturated with $H_2O$. The resulting powder was washed with 5% $NaHCO_3$ and $H_2O$, and recrystallized or precipitated from appropriate solvents.

*Evaluation of the Effect of Sulphur Trioxide Complex on Tyrosine Sulphation.*—Boc-Tyr-OH (14 mg, 0.05 mmol) was dissolved in DMF–pyridine (4:1; 0.5 ml), and DMF–$SO_3$ or pyridine–$SO_3$ complex (5 equiv.) was added. The mixture was stirred at 25 °C. The progress of the sulphation was periodically monitored by HPLC on a Nucleosil 7C8 column (4.6 × 250 mm), which was eluted with a gradient of MeOH (30–90% in 30 min) in 0.1% aqueous TFA at a flow rate of 1.0 ml/min [retention time ($t_R$) of Boc-Tyr($SO_3H$)-OH, 11.5 min; Boc-Tyr-OH, 17.1 min].

*Z-Tyr-Gly-Gly-Phe-Leu-OBzl.*—A TFA-treated sample of Z(OMe)-Gly-Gly-Phe-Leu-OBzl (1.00 g, 1.55 mmol) was dissolved in DMF (10 ml) containing TEA (0.22 ml, 1.55 mmol). The azide prepared from Z-Tyr-$NHNH_2$ (0.61 g, 1.86 mmol) and NMM (0.20 ml, 1.86 mmol) were added, and the mixture was stirred overnight. The product was purified by procedure A, followed by silica gel column chromatography (3.0 × 25 cm) using $CHCl_3$–MeOH (10:0.5) as eluant. The desired fractions were collected and concentrated and the residue was recrystallized from MeOH with ether to afford the crystalline *title compound* (0.95 g, 79%), m.p. 92–95 °C, $[\alpha]_D^{29}$ −14.9° (*c* 1.0, in MeOH); $R_{f1}$ 0.48, $R_{f3}$ 0.35 (Found: C, 65.7; H, 6.5; N, 8.8. $C_{43}H_{49}N_5O_9 \cdot 1/2H_2O$ requires C, 65.5; H, 6.3; N, 8.9%).

*H-Tyr($SO_3H$)-Gly-Gly-Phe-Leu-OH (Leu-enkephalin Sulphate).—By DMF–$SO_3$ complex.* Z-Tyr-Gly-Gly-Phe-Leu-OBzl (50 mg, 0.064 mmol) in DMF–pyridine (4:1, 1 ml) was treated with DMF–$SO_3$ complex (200 mg, 20 equiv.) at 30 °C for 2 h. The solvent was evaporated off at 30 °C, and the residue was treated with 10% $NH_4OH$ in an ice bath for 30 min so that the pH of the mixture was maintained at 8. The product was extracted with Bu$^n$OH, and the organic layer was washed with $H_2O$ and concentrated. The residue was treated with ether to afford a powder which was precipitated from DMF with ether to afford Z-Tyr($SO_3H$)-Gly-Gly-Phe-Leu-OBzl (55 mg, 100%; $R_{f1}$ 0.32) as a powder. Without further purification, the powder was dissolved in DMF (5 ml)–MeOH (10 ml) in the presence of a few drops of AcOH. Catalytic hydrogenation was then conducted at room temperature for 2 h in the presence of 5% Pd/C (*ca.* 1 g). The mixture was filtered, and the filtrate was concentrated *in vacuo*. The residue was dissolved in $H_2O$, washed with AcOEt, and lyophilized to afford a powder [Figure 2(*a*)] (42 mg, 100%), which was purified by HPLC on a column of YMC AM-323 (10 × 250 mm) with isocratic elution using 28% MeCN in 0.1M $AcOHN_4$ (pH 6.5) ($t_R$ of the desired product, 8.0 min) to afford pure Leu-enkephalin sulphate (27 mg, 66%), $R_{f5}$ 0.47. Amino acid ratios in a LAP digest: Tyr($SO_3H$) 0.79, Gly 1.86, Phe 0.94, Leu 1.00 (recovery of Leu, 95%). The HPLC retention time, 11.8 min [Figure 2(*b*)], was identical with that of the authentic sample; FTIR: $\nu_{max}$ 1 047 and 1 235 $cm^{-1}$.

*By Pyr–$SO_3$ complex.* Z-Tyr-Gly-Gly-Phe-Leu-OBzl (50 mg, 0.064 mmol) in DMF–pyridine (4:1; 1 ml) was treated with Pyr–$SO_3$ complex (204 mg, 20 equiv.) at 30 °C for 4 h. The product was purified as just described to give Z-Tyr($SO_3H$)-Gly-Gly-Phe-Leu-OBzl (55 mg, 100%; $R_{f1}$ 0.32), which was also subjected to catalytic hydrogenation (36 mg, 81%), followed by purification by HPLC as in the foregoing preparation to afford pure Leu-enkephalin sulphate (22 mg, 55%), $R_{f5}$ 0.47. Amino acid ratios in a LAP digest: Tyr($SO_3H$) 0.83, Gly 1.93, Phe 0.95, Leu 1.00 (recovery of Leu, 91%). The HPLC retention time was also 11.8 min (eluted under the same conditions as in Figure 2); FTIR: $\nu_{max}$ 1 048 and 1 236 $cm^{-1}$.

*Z(OMe)-Arg(Mts)-Phe-$NH_2$* (**1**).—The mixed anhydride prepared from Z(OMe)-Arg(Mts)-OH·CHA (6.83 g, 11.00 mmol) in DMF (50 ml) was added to an ice-chilled solution of a TFA-treated sample of Boc-Phe-$NH_2$ (2.91 g, 11.00 mmol) in DMF (20 ml) containing TEA (1.53 ml, 11.00 mmol). The mixture was stirred for 1 h, then the solvent was evaporated off *in vacuo*. The residue was purified by procedure A, followed by recrystallization from MeOH with ether to afford the *dipeptide* (**1**) (6.32 g, 86%). The physical constants and analytical data are in the Table, together with those of other intermediates.

*Z(OMe)-Met(O)-Arg(Mts)-Phe-$NH_2$* (**2**).—Z(OMe)-Met(O)-OSu [prepared from Z(OMe)-Met(O)-OH (3.29 g, 10.00 mmol)] in DMF (30 ml) and NMM (0.99 ml, 9.00 mmol) were added to an ice-chilled solution of a TFA-treated sample of (**1**) (6.00 g, 9.00 mmol) in DMF (50 ml) containing TEA (1.25 ml, 9.00 mmol). The mixture was stirred overnight. The product was purified by procedure A (extraction solvent: Bu$^n$OH), followed by recrystallization from MeOH with AcOEt to afford the *tripeptide* (**2**) (6.40 g, 88%).

*Z(OMe)-His-Met(O)-Arg(Mts)-Phe-$NH_2$* (**3**).—A TFA-treated sample of (**2**) (6.10 g, 7.50 mmol) was dissolved in DMF (50 ml) and the azide prepared from Z(OMe)-His-$NHNH_2$ (3.00 g, 9.00 mmol) in DMF (30 ml) and TEA (2.30 ml, 16.50 mmol) were added. The mixture was stirred overnight. The product was purified by procedure A (extraction solvent: Bu$^n$OH), followed by recrystallization from MeOH with AcOEt to afford the *tetrapeptide* (**3**) (7.10 g, 99%).

*Z(OMe)-Gly-His-Met(O)-Arg(Mts)-Phe-$NH_2$* (**4**).—A mixture of Z(OMe)-Gly-OSu (0.46 g, 1.90 mmol), NMM (0.18 ml, 1.60 mmol), and a TFA-treated sample of (**3**) (1.53 g, 1.60 mmol) in DMF (10 ml) containing TEA (0.22 ml, 1.60 mmol) was stirred overnight. The product was purified by procedure A (extraction solvent: Bu$^n$OH). The product was further purified by silica gel column chromatography (2 × 15 cm) using MeOH (10–20%)–$CHCl_3$ as eluant. The desired fractions were collected and concentrated, and the residue was treated with ether to afford the *pentapeptide* (**4**) (0.67 g, 42%).

*Boc-Tyr($Cl_2Bzl$)-Gly-His-Met(O)-Arg(Mts)-Phe-$NH_2$* (**5**).—A mixture of Boc-Tyr($Cl_2Bzl$)-OSu (0.53 g, 0.99 mmol), NMM (0.11 ml, 0.99 mmol), and a TFA-treated sample of (**4**) (0.83 g, 0.82 mmol) in DMF (10 ml) containing TEA (0.11 ml, 0.82 mmol) was stirred overnight. The product was purified by procedure A (extraction solvent: Bu$^n$OH), followed by precipitation from DMF with AcOEt–ether to afford the *hexapeptide* (**5**) (0.77 g, 74%).

*Boc-Asp(OChp)-Tyr($Cl_2Bzl$)-Gly-His-Met(O)-Arg(Mts)-Phe-$NH_2$* (**6**).—A mixture of Boc-Asp(OChp)-OSu [prepared from Boc-Asp(OChp)-OH (3.08 g, 9.35 mmol)], NMM (0.94 ml, 8.50 mmol), and a TFA-treated sample of (**5**) (10.80 g, 8.50 mmol) in DMF (100 ml) containing TEA (1.19 ml, 8.50 mmol) was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF with ether to afford the *heptapeptide* (**6**) (11.20 g, 89%).

*Z(OMe)-Asp(OChp)-Asp(OChp)-Tyr($Cl_2Bzl$)-Gly-His-Met(O)-Arg(Mts)-Phe-$NH_2$* (**7**).—A mixture of Z(OMe)-Asp(OChp)-OSu [prepared from Z(OMe)-Asp(OChp)-OH·CHA (2.42 g, 4.90 mmol)], NMM (0.45 ml, 4.10 mmol), and a TFA-treated sample of (**6**) (6.10 g, 4.10 mmol) in DMF (50 ml) containing TEA (0.57 ml, 4.10 mmol) was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF with AcOEt to afford the *octapeptide* (**7**) (6.60 g, 92%).

*Z(OMe)-Ser(Bzl)-Asp(OChp)-Asp(OChp)-Tyr($Cl_2Bzl$)-Gly-His-Met(O)-Arg(Mts)-Phe-$NH_2$* (**8**).—A mixture of Z(OMe)-

**Table.** Physical constants and analytical data of the protected leucosulfakinin-II (**9**) and its intermediates.

| Peptide | $R_{f1}$ | M.p. (*t*/°C) | $[\alpha]_D^{23}$/° (*c* 1) | Formula | % Found (Required) C | H | N |
|---|---|---|---|---|---|---|---|
| (**1**) | 0.62 | 95–99 | −8.0 (MeOH) | $C_{33}H_{42}N_6O_7S\cdot 3/2H_2O$ | 57.3 (57.1 | 6.3 6.3 | 12.1 12.1) |
| (**2**) | 0.62 | 117–119 | −11.0 (MeOH) | $C_{38}H_{51}N_7O_9S_2\cdot 2H_2O$ | 54.0 (53.7 | 6.2 6.3 | 11.6 11.5) |
| (**3**) | 0.47 | 133–137 | −13.0 (MeOH) | $C_{44}H_{58}N_{10}O_{10}S_2\cdot 2H_2O$ | 53.3 (53.5 | 6.5 6.3 | 14.0 14.2) |
| (**4**) | 0.42 | 132–136 | −10.9 (DMF) | $C_{46}H_{61}N_{11}O_{11}S_2\cdot 7/2H_2O$ | 51.9 (51.6 | 6.3 6.4 | 13.9 14.4) |
| (**5**) | 0.51 | 140–141 | −6.0 (DMF) | $C_{58}H_{74}Cl_2N_{12}O_{12}S_2\cdot 5H_2O$ | 51.2 (51.4 | 5.8 6.2 | 12.3 12.4) |
| (**6**) | 0.50 | 141–144 | −10.2 (DMF) | $C_{69}H_{91}Cl_2N_{13}O_{15}S_2\cdot 5H_2O$ | 52.5 (52.9 | 6.2 6.5 | 12.0 11.6) |
| (**7**) | 0.49 | 137–140 | −11.9 (DMF) | $C_{84}H_{108}Cl_2N_{14}O_{19}S_2$ | 56.3 (56.4 | 6.3 6.3 | 11.0 11.0) |
| (**8**) | 0.61 | 140–143 | −12.9 (DMF) | $C_{94}H_{119}Cl_2N_{15}O_{21}S_2\cdot 3H_2O$ | 56.6 (56.9 | 6.25 6.35 | 10.8 10.8) |
| (**9**) | 0.42 | 192–195 | −12.8 (DMF) | $C_{90}H_{116}Cl_2N_{16}O_{20}S_2\cdot 5H_2O$ | 55.1 (54.95 | 6.4 6.5 | 11.5 11.4) |

Ser(Bzl)-OSu [prepared from Z(OMe)-Ser(Bzl)-OH·CHA (0.20 g, 0.43 mmol)], NMM (0.03 ml, 0.29 mmol), and a TFA-treated sample of (**7**) (0.50 g, 0.29 mmol) in DMF (50 ml) containing TEA (0.04 ml, 0.29 mmol) was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF with AcOEt to afford the *nonapeptide* (**8**) (0.45 g, 91%).

*pGlu-Ser(Bzl)-Asp(OChp)-Asp(OChp)-Tyr($Cl_2$Bzl)-Gly-His-Met(O)-Arg(Mts)-Phe-$NH_2$* (**9**).—A mixture of pGlu-OSu [prepared from pGlu-OH (0.06 g, 0.47 mmol)], NMM (0.05 ml, 0.47 mmol), and a TFA-treated sample of (**8**) (0.69 g, 0.36 mmol) in DMF (10 ml) containing TEA (0.05 ml, 0.36 mmol) was stirred overnight. The product was purified by procedure B, followed by recrystallization from DMF with AcOEt to afford the *decapeptide* (**9**) (0.63 g, 94%). Amino acid ratios in a 6M HCl hydrolysate: Asp 2.03, Ser 0.82, Glu 0.96, Gly 1.00, Met + Met(O) 0.73, Tyr 1.01, Phe 1.00, His 0.91, Arg 1.02 (recovery of Phe, 92%).

*pGlu-Ser-Asp-Asp-Tyr-Gly-His-Met-Arg-Phe-$NH_2$ (Non-sulphated Leucosulfakinin-II).*—The decapeptide (**9**) (50 mg, 0.027 mmol) in DMF–pyridine (4:1, 1 ml) was treated with DMF–$SO_3$ complex (166 mg, 40 equiv.) and EDT (102 µl, 40 equiv.) at 30 °C for 2 h, then the solvent was removed by evaporation at 30 °C. The residue was treated with $H_2O$, and the resulting powder was precipitated from DMF with ether to afford the reduced, protected peptide (yield 45 mg, 91%), $R_{f1}$ 0.54. Amino acid ratios in a 4M MSA hydrolysate: Asp 1.96, Ser 0.73, Glu 0.84, Gly 1.06, Met 0.87, Tyr 0.95, Phe 1.00, His 0.82, Arg 1.00 (recovery of Phe, 79%); FAB MS, *m/z* 1 859.8 $(M + H)^+$]. The reduced protected peptide was then deprotected with 1M TFMSA–thioanisole/TFA (5 ml) in the presence of *m*-cresol (200 µl, 1.85 mmol) and EDT (100 µl, 1.06 mmol) in an ice bath for 3 h, and ether was added. The resulting powder was collected by centrifugation, and dissolved in MeOH–$H_2O$ (0.5 ml–2 ml) containing 2-mercaptoethanol (20 µl) and NMM (50 µl) in an ice bath. After 15 min, the solution was acidified with AcOH to pH 4, and applied to a column of Sephadex G-10 (2.4 × 60 cm) which was eluted with 2M AcOH. Each fraction (6 ml) was monitored by the UV absorption at 275 nm, the desired fractions (nos. 17–25) were collected, and the solvent was removed by lyophilization to afford a powder [Figure 3(*a*)] (17 mg, 57%). Subsequent purification was performed by reverse-phase HPLC on a YMC AM-323 column (10 × 250 mm). Portions of the above crude sample (*ca.* 2 mg each) were applied to the column, which was eluted with a linear gradient of MeCN (20–40% in 30 min) in 0.1% TFA at a flow rate of 2.5 ml/min. The eluant corresponding to the main peak ($t_R$ 13.6 min, monitored by UV absorption at 275 nm) was collected and lyophilized to give a white fluffy powder (12 mg combined from all samples, 40%), $[\alpha]_D^{23}$ −6.2° (*c* 0.5, in 0.1% TFA), $R_{f4}$ 0.40; HPLC $t_R$ 17.8 min [Figure 3(*b*)]. Amino acid ratios in a 6M HCl hydrolysate: Asp 1.99, Ser 0.93, Glu 0.98, Gly 0.99, Met 0.91, Tyr 1.01, Phe 1.00, His 0.97, Arg 0.98 (recovery of Phe, 72%); FAB MS, *m/z* 1 237.2 $(M + H)^+$.

*pGlu-Ser-Asp-Asp-Tyr($SO_3H$)-Gly-His-Met-Arg-Phe-$NH_2$ (Leucosulfakinin-II).*—A gel-filtered sample of the foregoing non-sulphated decapeptide (30 mg, 0.024 mmol) was dissolved in DMF (1 ml) together with imidazole (49 mg, 30 equiv.) and phenol (68 mg, 30 equiv.), then $Bu^tPh_2SiCl$ (167 µl, 30 equiv.) was added and the solution was stirred in an ice bath for 2.5 h. Ether was added, and the precipitated oil was washed with ether. The product ($R_{f4}$ 0.50) was purified by gel-filtration on Sephadex LH-20 (2.4 × 78 cm) with DMF as eluant. The desired fractions (7 ml each, tube nos. 25–51, monitored by UV absorption at 280 nm) were combined and the solvent was removed by evaporation. The residue was dissolved in 20% pyridine in DMF (2.5 ml), then EDT (23 µl, 10 equiv.) and DMF–$SO_3$ complex (183 mg, 50 equiv.) were added and the mixture was stirred at 30 °C for 36 h. The solution was applied to a column of Sephadex LH-20 (2.4 × 78 cm), which was eluted with DMF as stated above. The desired fractions (tube nos. 17–22) were combined and the solution was concentrated to 1 ml. This solution was treated with $Bu_4NF\cdot 3H_2O$ (168 mg, 20 equiv.) in the presence of EDT (47 µl, 20 equiv.) in an ice bath for 1 h, then at 30 °C for 1 h. With ice cooling, 2M AcOH (5 ml) was added and a small amount of insoluble material was removed by centrifugation. The supernatant liquid was applied to a column of Sephadex G-10 (2 × 45 cm), which was eluted with 2M AcOH. The fractions corresponding to the front peak (5 ml each, tube nos. 14–17, monitored by UV absorption at 275 nm) were combined and the solvent was removed by lyophilization to give a powder (11.1 mg, 35%) [Figure 4(*a*)]. The product was further purified by HPLC on a YMC AM-312 column (6 × 150 mm). Portions of the above crude sample (*ca.*

0.5 mg each) were applied to the column, which was eluted with a linear gradient of MeCN (12–25% in 30 min) in 0.1M $AcONH_4$ (pH 6.5) at a flow rate of 1 ml/min. The eluate corresponding to the main peak ($t_R$ 15.5 min, monitored by UV absorption at 265 nm) was collected and lyophilized to give a white fluffy powder (3.3 mg combined yield, 11%), $[\alpha]_D^{22}$ −26.0° (*c* 0.2, in 0.1M AcOH), $R_{f4}$ 0.25; HPLC $t_R$ 15.2 min [Figure 4(*b*)]. Amino acid ratios in a 0.2M $Ba(OH)_2$ hydrolysate: Asp 1.85, Ser 0.43, Glu 1.08, Gly 0.98, Met 0.90, Tyr($SO_3H$) 0.78, Phe 1.00, His 0.71, Arg not determined (recovery of Phe, 96%); FTIR, $\nu_{max}$ 1 047 and 1 259 $cm^{-1}$; FABMS, *m/z* 1 338.9 $(M + Na)^+$. The total yield was 5% when basically the same experiment was conducted using the Pyr–$SO_3$ complex (50 equiv.) as a sulphating agent instead of the DMF–$SO_3$ complex.

## Acknowledgements

We thank Professor N. Fujii of Kyoto University for amino acid analysis, and Dr. H. Tanaka of Fujisawa Pharmaceutical Ind., Co. Ltd. for FABMS measurements.

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*Paper* 9/04801D
*Received* 9*th November* 1989
*Accepted* 18*th December* 1989