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Bicyclic Turned Dipeptide (BTD) as a β-Turn Mimetic; its Design, Synthesis and Incorporation into Bioactive Peptides

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Abstract: After reviewing the development of β-turn mimetics briefly, design, synthesis and incorporation into bioactive peptides of BTD will be mentioned. The biological activity of the BTD containing peptides and synthesis of some BTD derivatives will also be described.

§ 1 Introduction

α-Helix, β-sheet and β-turn are three major secondary structures found in polypeptides and proteins. Among them the β-turn is also frequently found in bioactive peptides. It functions to reverse the direction of chain propagation. Since a β-turn is composed of only four amino acid residues, it is an appropriate target for an organic chemist to design the mimetics.

The stereochemistry of gramicidin S (GS) was the start line of our studies on β-turns. The conformation of GS was established based on the analysis of the nmr spectrum by Stern et al.[1], and the conformation activity relation was suggested by Schwyzer and Kato et al.[2] What attracted our interest was the motive force that induces the cyclic decapeptide to take on the β-sheet conformation with two β-turns at the ends of the sheet. The preference of the tetrapeptide sequence at the corner position was considered to be the key to induce the β-sheet conformation of GS[3], and the concept was verified by structure activity relationships of some GS analogues and by measurement of the β-turn preference of the corresponding tetrapeptide sequence at the corners by means of CD spectra of their chromophoric derivatives[4]. The extremely high preference for a β-turn caused by covalent fixing the turn conformation was considered to stabilize the GS conformation efficiently and to give an analogue of high biological acitivity. This was one of our motivations to design BTD. On the other hand, the relation of peptide secondary structure to the CD spectra is well established for α-helix and β-sheet[5]. However, the characteristic CD spectrum for a β-turn seems not to be established. Therefore, the synthesis of an appropriate compound with a fixed β-turn conformation would shed light on the

problem[6]. There was another motivation to stimulate us to design BTD. It is the work by Freidinger et al.[7] who synthesized an analogue of LRF with a restricted β-turn conformation at its 6,7-positions by incorporation of a lactam derivative of leucine. Their paper suggested that incorporation of a dipeptide unit with a fixed β-turn conformation into linear bioactive peptides would give information concerning their active conformation and, if lucky, highly active and selective analogue would be obtained.

After a number of unsuccessful trials to achieve a BTD-like skeleton containing carbon atoms only except the lactam moiety, it was recognized that incorporation of a sulphur atom in the nucleus would facilitate the synthesis without affecting significaltly the conformation. The synthetic route to penicillin by Sheehan et al.[8] was the model scheme for the synthesis of BTD. Here, its design, synthesis, incorporation into bioactive peptides and their biological activity will be described although part of the results were reported in the form of short communication and symposium proceedings.

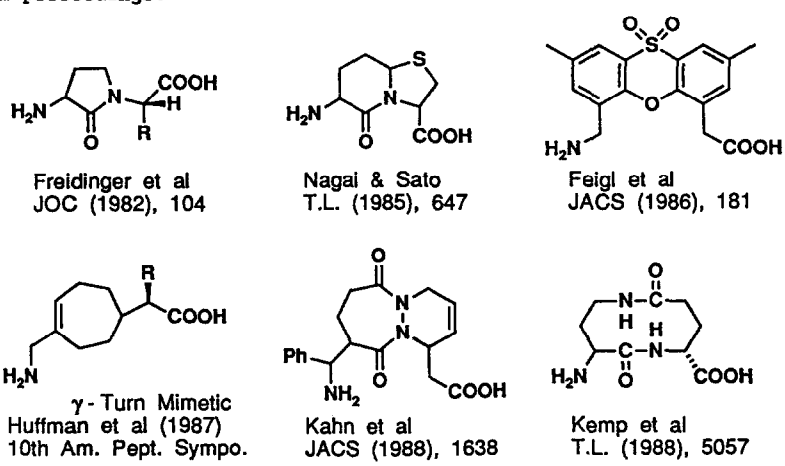


Fig. 1 A list of the structures of turn mimetics

After publication of our initial paper several turn mimetics were reported. Their structures are listed in Fig. 1. Their chemical structures and conformations were well confirmed by analysis of the nmr spectra. According to the introductory part of these papers, the major concern of their designing such mimetics consists in the elucidation of bioactive conformation of the peptides into which the mimetics will be incorporated. However, so far we know, there seems to be few reports of their incorporation into bioactive peptides despite their initial intention for the design and synthesis.

§ 2 Synthesis of BTD

The scheme of BTD synthesis is shown in Fig. 2. Phthaloyl-L-glutamic anhydride 1

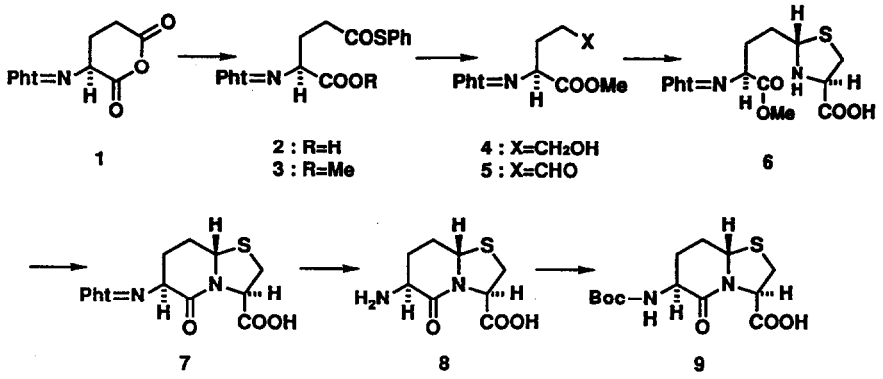


Fig. 2 Synthetic scheme of BTD and its N-protected derivatives

was treated with thiophenol to give the γ-half thioester 2, which was then converted to the α-methyl ester 3 by treatment with diazomethane. Raney-nickel reduction of 3, followed by PCC oxidation afforded the γ-aldehyde 5. This compound 5 is the key intermediate of the BTD ,synthesis. Overnight reaction of L-cysteine with 5 in aqueous ethanolic solution at room temp. gave compound 6 as white voluminous precipitates. The precipitates collected by filtration were heated overnight at 70 ℃ without further purification. The reaction product was purified by chromatography and recrystallization from MeOH to give the desired compound 7. The structure of 7 was confirmed by uv, ir and nmr (including NOE) spectra and also by elemental analysis[9]. The phthaloyl group of 7 was removed by treatment with hydrazine hydrate in MeOH to give free BTD 8, which in turn was converted to the Boc derivative 9 by the usual procedure using di-*t*-butyl dicarbonate. The Boc-BTD 9 can be used generally as a dipeptide building block in peptide synthesis including solid phase synthesis.

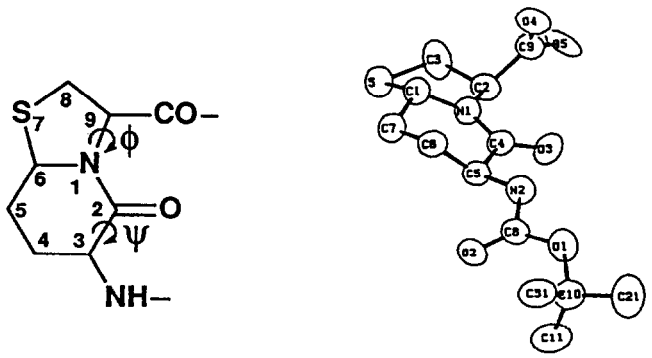
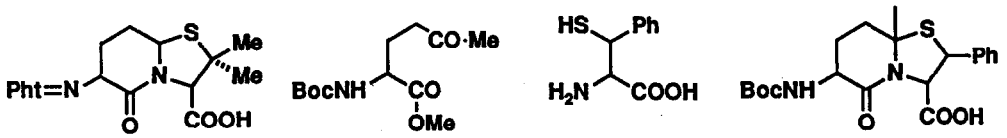


Fig. 3 Conformation of Boc-BTD by X-ray analysis

Boc-BTD provided nice crystals by evaporating the EtOAc/MeOH solution which were subjected to X-ray crystallographic analysis. The result is reproduced in Fig. 3. The dihedral angles were -161 ° for ψ and -69 ° for φ in the crystalline state, which proved that the conformation of BTD corresponds to type 2' β-turn as

expected[10].

The BTD skeleton can be constructed from glutamic acid and cysteine. If a β-substituted cysteine instead of cysteine is used 8-substituted BTD can be synthesized. Penicillamine (β,β-dimethylcysteine) was treated with compound 5 to give 8,8-dimethyl BTD 10. The yield, however, was poor and not improved even by a number of changes in the reaction conditions. Similarly, 8-phenylBTD derivative 13 was synthesized by the reaction of compound 11 with β-mercaptophenylalanine 12[11]. The synthesis of compound 12 was reported as a short communication[12]. The details of the synthesis are not described here due to space limitation.



10 11 12 13

Fig. 4 Structures of 8-substd. BTD and the building blocks

§ 3. BTD incorporated analogues of bioactive peptides — the synthesis and the biological activity.

BTD was incorporated into the bioactive peptides listed below; enkephalin, GS, LRF, somatostatin and growth hormone releasing factor (GRF) by ourselves. In addition, BTD was also icorporated into HIV-1 protease by B. H. Kent et al[13]. The analogues synthesized was subjected to bioassay in due course. The method of their synthesis and their biological activity will be described hereafter.

a) **Enkephalin analogue:** The Gly^2-Gly^3 sequence in enkephalin was replaced with BTD. The position of BTD incorporation was selected based on the result of X-ray crystal analysis of [Leu^5]enkephalin by Smith and Griffin[14]. The synthesis was carried out according to the scheme shown in Fig. 5 by classical solution method. Leucine amide was treated with Boc-Phe-OH in DMF using WSCD as the coupling agent to yield Boc-Phe-Leu-NH_2 **14**, which was deprotected as usual and treated with Pht>BTD-OH **7** similarly to afford Pht>BTD-Phe-Leu-NH_2 **15**. After removal of the Pht-group, it was then treated with Boc-Tyr-OSu in DMF to yield Boc-Tyr-BTD-Phe-Leu-NH_2 **16**, which is the Boc-derivative of the desired enkephalin analogue. The analogue synthesized, [BTD^{2-3},Leu^5]enkephalin amide **17**, exhibited very weak activity in inhibiting contraction of the guinea pig ileum by electrical stimulation (20% inhibition at the concentration of 2×10^{-5}M). The inhibition was reversible by treatment with naloxone (1×10^{-5}M). A binding assay was also carried out using the receptor from the rat brain and [^{3}H]-dalamid as the radioactive ligand. From the experiment the IC_{50}-value of the analogue was estimated to be 2.3×10^{-5}M. The presence of a thiazolidine-4-carboxylic acid moiety (Pro-like structure) at the 3-position seems to be the reason for weak

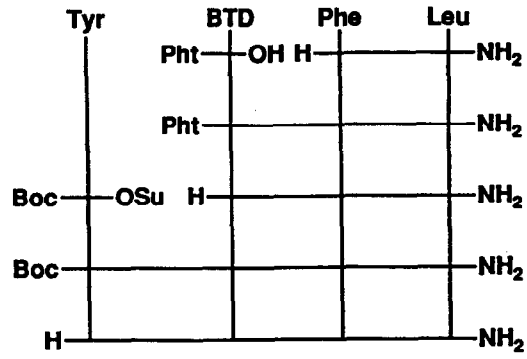


Fig. 5 Scheme for the [BTD$^{2-3}$, Leu^{5}] enkephalin amide synthesis

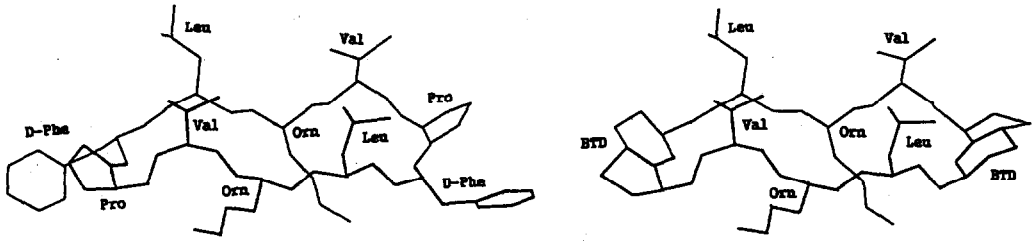


Fig. 6 Conformation of native GS and [BTD]GS

activity of the analogue because [Pro^{3},Leu^{5}]enkephalin was reported to have very weak opioid activity that is less than 1/1000 of [Met^{5}]enkephalin[15].

b) Gramicidin S analogue: Since the synthesis of this analogue was already reported in detail[16], only the results are described briefly. The conformation of native G.S. and the BTD incorporated analogue are shown in Fig. 6. They exhibited the same antibacterial activity. The result indicates that G.S. maintains the proper

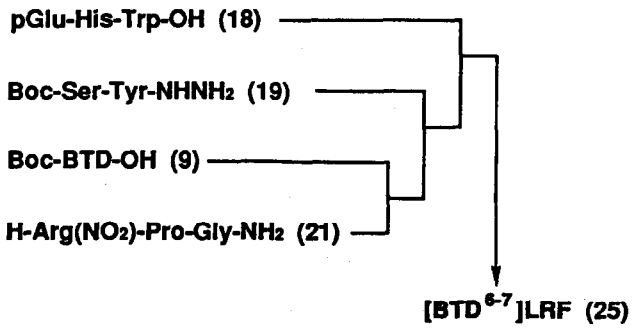


Fig. 7 Scheme of [BTD$^{6-7}$]LRF synthesis

solution conformation at the site where the antibiotic activity is exhibited, too.

c) LRF analogue: LRF has the structure of <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. It was suggested to have a turn conformation at the Gly-Leu (6-7) sequence based on the results of energy calculation and structure activity relationship[17,18]. The pioneering work of Freidinger et al.[7] also supports the hypothesis. Accordingly, BTD was incorporated in place of the Gly-Leu sequence. The synthetic scheme of the BTD containing analogue is shown in Fig. 7. The synthesis was carried out by a similar strategy to that reported by Fujino et al for the synthsis of LRF[19]. The target molecule was divided into four fragments, pGlu-His-Trp-OH 18, Boc-Ser-Tyr-$NHNH_2$ 19, Boc-BTD-OH 9 and H-Arg(NO_2)-Pro-Gly-NH_2 21. Compounds 9 and 21 were coupled to give Boc-pentapeptide 22. Removal of the Boc-group followed by coupling with the azide derived from 19 afforded Boc-heptapeptide 23. Deprotection of 23 followed by coupling with 18 using DCC/HONb afforded the protected decapeptide amide 24, which has a nitro group on the Arg residue. Treatment of 24 with liquid HF afforded the desired analogue of LRF 25. The analogue 25 was purified by combination of ion-exchange chromatography and reverse phase HPLC. The purity of 25 was confirmed by TLC, HPLC, amino acid and elemental analyses. The biological activity of 25 was assayed by *in vitro* and *in vivo* experiments. The result of the latter experiment are reproduced in Fig. 8. [BTD]LRF retains high potency (EC_{50} = $3x10^{-9}$M) and proves the existence of a β-turn at 6-7 position in the bioactive conformation although the potency is 1/10 of LRF. The slightly weaker activity of [BTD]LRF seems due to the lack of the side-chain, which would result in less affinity to the receptor.

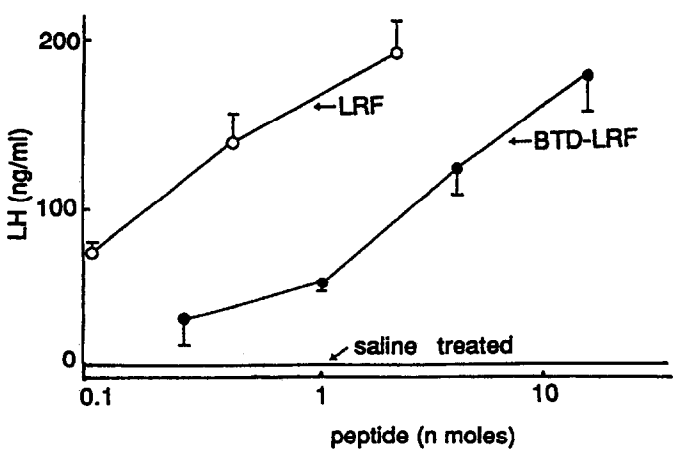


Fig. 8 Results of bioassay of native LRF and [BTD]LRF

d) GRF analogue: Since the synthesis and the biological activity of BTD incorporated analogues of GRF were reported in detail previously[20], it is not mentioned here. It seems, however, worthwhile to mention that the synthesis was carried out by solid phase methods using Boc-HF strategy because it proves that BTD can be incorporated into big peptides tolerating the reaction conditions of solid phase synthesis.

e) The other analogues containing BTD: In addition to the analogues mentioned above , BTD was incorporated into a cyclic hexapeptide analogue of somatostatin, which has the structure cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe)[21]. The D-Trp-Lys sequence was replaced with BTD because the sequence was considered to prefer β-turn conformation. The BTD containing analogue did not exhibit any biological activity. Compound (13), 6-methyl-8-phenyl-BTD, was incoporated into the N-terminal tetrapeptide of dermorphin, Tyr-D-Ala-Phe-Gly-NH_2, which is known to maintain potent activity[11,22]. The D-Ala-Phe sequence was replaced with the 8-phenyl-BTD. The BTD containing analogue also did not show any binding activity to the opioid receptor of rat brain. The experimental details of the synthesis of these analogues are not described because of space limitation. Besides our own work, Kent et al incorporated BTD into HIV protease. The chemically synthesized analogue [$BTD^{16-17,116-117}$ $Aba^{67,95,167,195}$]HIV-1 protease was reported to show high enzymatic activity[13].

§ 4. Prospective consideration based on the experience described above.

Seven BTD containing analogues of bioactive polypeptides and proteins were mentioned above. Among them, three showed potent activity, and the others showed only very weak or no activity. The results can be interpreted as follows. The role of amino acid residues involved at the turning positions can be in two ways; i) induction of turned conformation in collaboration with the adjacent residues, and ii) possible interaction with the receptor (in a broad sense). In the latter case the side-chain functionality may play significant roles for the receptor binding. BTD is considered a good substitute for the first role, but it seems not to be useful for the cases where the turn position itself plays some role for interaction with the receptor site. As shown in the cases of enkephalin, small cyclic somatostatin and dermorphin, such small bioactive peptides seem to bind to the receptor with a high proportion of the molecular surface and subtle changes in the functionality and conformation would be critical for expression of biological acitivity. In contrast, in most of the cases of bigger peptides, the turn position residues would play the single role of conformation restriction and are not involved in direct interaction with the receptor. In such cases, potent activity can be expected for the BTD incorporated analogues. [BTD^{8-9}]GRF seems to be an unlucky case where the BTD incorporated position would be involved in the interaction with the receptor by

chance in spite of the big size of molecule. This concept will be helpful for selecting the target bioactive peptide into which BTD will be incorporated. If used with such proper consideration, BTD seems to be a useful tool for elucidating bioactive conformation, and in lucky cases highly potent and selective analogues would be obtained.

Acknowledgements: We are grateful to Dr. Shao-Yao Ying of The Salk Institue for the bioassay of LRF analogue, to Dr. Y. Kudo and Dr. M. Maruyama of Mitsubishi-Kasei Institute of Life Sciences for bioassay of opioid peptide analogues, to Dr. T. Kanmera of Mitsubish Kasei Ltd. Reseach Center for HF treatment procedure, and Dr. T. Matsuzaki and his collaborators of the same Center for X-ray crystal analysis of Boc-BTD and the members of the Analytical Department of the Center for elemental analyses.

Experimental

Melting points were measured with YANACO MP micro melting point apparatus (Yanagimoto). Specific rotations were determined with JASCO DIP-360 digital polarimeter. Infrared spectra were measured with Hitachi 260-50 Infrared Spectrophotometer. Mass spectra were measured with JMS-H100 mass spectrometer (JEOL). Nmr spectra were measured with FX-100 NMR spectrometer (JEOL).

Synthesis of BTD

Synthesis of γ-thiophenyl N-phthalyl-L-glutamate (2)

To N-phthalyl-L-glutamic anhydride (1, 5.2g) was added dioxane (20ml) , and then thiophenol (1.32g) and dicyclohexylamine (DCHA, 1.90g) were added under stirring. Crystals of N-phthaloyl-glutamic anhydride disappeared in a few min. Stirring was continued for 72hr. Exhaustive evaporation of the solvent *in vacuo* at 50° afforded viscous, slightly yellow oily residue, which was triturated with Et_2O (50ml) and kept overnight in a refrigerator. The crystals formed were collected by filtration and drying gave the DCHA-salt of product (4.7g, 85% yield). M.p. 147-156° .

The DCHA-salt (4.5g) was treated with 10% citric acid (1.5N, 40ml) and EtOAc (20ml), and stirred vigorously. After separation of the organic layer, the aqueous layer was extracted again with EtOAc (30ml). Combined organic layer was washed with water (20ml) and dried over Na_2SO_4. Evaporation of the solvent after removal of the desicant afforded viscous oily product, which crystallized slowly on leaving. When not crystallized, seeding of small amount of crystals was effective. The yield was 3.0g (quantitative). M.p. 182-191°, $[\alpha]_D$ -36.0° (c 0.98, DMF, t=23°). Elem. Anal. $C_{19}H_{15}NO_5S$ requires C:61.78, H:4.09, N:3.79, S:8.68%; Observed C:61.85, H:4.03, N:3.87, S:8.57%.

Synthesis of α-methyl, γ-thiophenyl N-phthalyl-L-glutamate (3)

The compound (2, 2.7g) was suspended in EtOAc (20ml). Ethereal diazomethane solution was added dropwise to the suspension until complete dissolution of solid material and slight yellow coloring of the solution. Evaporation of the reaction mixture afforded viscous oil (2.9g, yield 100%).

Synthesis of α-methyl N-phthalyl-L-glutamate γ-carbinol (4)

Raney-Ni (90g) suspended in EtOH (300ml) with a small amount of AcOH (0.6ml) was refluxed on a water-bath. Compound (3, 6g) dissolved int EtOH (50ml) was dropped into the refluxing suspension in the course of 20min. After addition of the diester finished, refluxing was continued for 2hr. In the final course, the reaction mixture was monitored by tlc (silica, $CHCl_3$: MeOH = 99:1), and the refluxing was stopped when the starting material disapeared. After cooling Raney-Ni was filtered off and washed with EtOH (30ml), and the combined filtrate and washings were evaporated *in vacuo*. The oily residue was dissolved in $CHCl_3$ and passed through a column of Florisil to remove insoluble material and then subjected to chromatography (silica 200g) using a mixture of $CHCl_3$ and MeOH (1, 2 and 4%) for elution. The fractions showing tlc-spot of the desired product (Rf=0.25, silica, $CHCl_3$: MeOH = 99:1) were collected, and evaporated to give oily product (3.1g, yield 70%). nmr ($CDCl_3$) δ ppm 1.60 (2H, m.), 2.30 (2H, m.), 3.56 (2H, t.), 3.66 (3H, s.), 4.83 (1H, dd.), 7.74 (4H, m.).

Synthesis of α-methyl N-phthalyl-L-glutamate γ-aldehyde (5)

To a stirred solution of PCC (1.2g) in CH_2Cl_2 (7.5ml) was added compound (4, 1.0g) dissolved in CH_2Cl_2 (6ml) under N_2-atmosphere. Stirring was continued for 2hr. monitoring disappearance of the starting material by tlc (silica, $CHCl_3$: MeOH = 98:2). By addition of anhydrous Et_2O (30ml) dark brown ppt. were formed. The supernatant was decanted off, and the sticky ppt. were triturated with Et_2O twice. The combined Et_2O layer was passed through a column of Florisil and the column was washed with Et_2O. UV absorbing fractions were collected and evaporated to give oily residue (0.6g, 60% yield). tlc Rf=0.29 (silica, $CHCl_3$: MeOH = 98:2). nmr ($CDCl_3$) δ ppm 2.50 (4H, m.), 3.69 (3H, s.), 4.80 (1h, dd.), 7.77 (4H, m.), 9.50 (1H, s.).

Synthesis of the thiazolidine compound (6)

Anhydrous NaOAc (500mg) was added to L-cysteine.HCl (425mg) solution dissolved in degassed water (6ml) under N_2-atmosphere. Compound (5, 600mg) dissolved in degassed EtOH (10ml) was added thereto under stirring. Stirring at room temp. was continued for 8hr. under N_2-atmosphere. About 10min. after starting the reaction, the reaction mixture became turbid. If not turbid slight warming (ca. 40°) is advisable. The reaction mixture was diluted with water (30ml), left several hours at room temp. to wait growing of the ppt., which were then filtered, washed with a small

amount of water and dried. The dried crude product was powdered with a mortar, a small volume of $CHCl_3$ was added to dissolve the unreacted starting material, and filtered. The dried product weighed 500mg (yield 60%). tlc Rf=0.60 (silica, BuOH: AcOH: H_2O = 4:1:2). This product was used for the next step without further purification.

Synthesis of N-phthalyl-BTD (7)

The thiazolidine compound (6, 1.0g) was dissolved in DMF (20ml), and heated under N_2-atmosphere in an oil-bath of 90°. The progress of reaction was monitored by tlc (silica, BuOH: AcOH: H_2O = 4:1:2). When the spot of starting material disappeared, heating was stopped. Evaporation of DMF from the reaction mixture afforded oily residue, all of which crystallized on seeding of a few crystals. A small volume of EtOAc (5ml) was added, triturated and kept in a refrigerator more than 1hr. to wait crystals growing. Crystals were filtered and washed with cold EtOAc and dried (A). Combined filtrate and washings were extracted with satd. $NaHCO_3$, which was acidified with 10% citric acid and extracted with EtOAc. The organic layer was washed with satd. NaCl and dried over Na_2SO_4. Evaporation of the solvent and recrystallization of the residue afforded the cystals of product (B). Combined weight of (A) and (B) were 730mg (80% yield). M.p. 266-268° (dec), $[\alpha]_D$ -249° (c 1.0, DMF, t=24°). tlc Rf=0.23 (silica, $CHCl_3$: MeOH: AcOH = 91:8:1). uv (MeOH) λ max (ε) 297 nm (sh.1770), 291 (1910), 237 (sh.8760), 217 (49200); ir(KBr) ν max cm^{-1} 3280 (carboxyl), 2930 (CH-stretching), 2560 (carboxyl), 1780, 1760, 1720, 1650 (4 carbonyls); ^{1}H-nmr (DMSO-d_6) δ ppm: 1.8-2.6 (4H, br.m.), 3.0-3.6 (2H, d.q.), 4.6-5.6 (3H, m.), 7.89 (4H, s.); Elem. Anal. $C_{16}H_{14}N_2O_5S$ requires C:55.48, H:4.07, N:8.09, S:9.25%; Observed C:55.22, H:4.06, N:8.22, S:9.26%.

Synthesis of BTD (8)

Compound (7, 300mg) was dissolved in EtOH (4ml) by slight heating. A solution of hydrazine hydrate (210 μl) in EtOH (8ml) was added to the above solution. The reaction mixture was heated under reflux for 1hr. It was evaporated *in vacuo*, and water (5ml) was added to the residue, the pH was adjusted to 4.0 and kept in a refrigerator for 30min. After removal of the ppt. the filtrate was passed through a column of Dowex- I(OH^- form), the column was washed with water until neutral. The column was eluted with 1M AcOH and ninhydrin positive fractions were collected and evaporated. Yield 85%. M.p. >280° $[\alpha]_D$ -273.8° (c 1.0, H_2O, t=25°). Elem. Anal. $C_8H_{12}N_2O_3S$ requires C:44.43, H:5.59, N:12.95, S:14.83%; Observed C:44.13, H:5.39, N:13.11, S:14.53%.

Synthesis of N-Boc-BTD (9)

BTD (8, 108mg) was dissolved in 0.2N-NaOH (2.5ml). Boc_2O (120mg) dissolved in

dioxane (2.5ml) was added to the solution in an ice-bath. The reaction mixture was stirred at room temp. for 2 hr. Water (15ml) was added to the reaction mixture, acidified with 10% citric acid and extracted with EtOAc (50ml), which was washed with 10% citric acid and satd. NaCl, and dried over Na_2SO_4. Evaporation of the filtered solution afforded oily residue, which was then triturated with EtOAc-pentane mixture in a deeply cooled bath of dry-ice and EtOH. By pipetting off the supernatant crystalline powder of the product was obtained. Yield: 134mg (85%), m.p.151-157°, $[\alpha]_D$ -214° (c 1.0, MeOH t=24°). Elem. Anal. $C_{13}H_{20}N_2O_5S$ requires C:49.35, H:6.37, N:8.85, S:10.13%; Observed C:49.21, H:6.26, N:8.77, S:10.29%.

Synthesis of Fmoc-BTD

BTD (8, 87mg) was dissolved in 10% $NaHCO_3$ (1.0ml). Under ice-cooling Fmoc chloride (100mg) in dioxane (0.75ml) was added and stirred for 3hr. The reaction mixture was poured onto cold water (20ml), then the clear aqueous solution was acidified with 4N HCl under ice-cooling to pH 2.0, and extracted with EtOAc 3 times. The combined EtOAc extracts was washed with satd. NaCl and dried over Na_2SO_4. Evaporation of the solvent afforded oily residue (159mg). Trituration of the residue with small amount of EtOAc gave crystals (71mg, yield 40%). M.p. 216-219°, $[\alpha]_D$ -146.7° (c 1.0, DMF, t=22°). tlc Rf=0.14 (silica, $CHCl_3$:MeOH:AcOH = 91:8:1). Elem. Anal. $C_{23}H_{22}N_2O_5S$ requires C:62.99, H:5.05, N:6.38, S:7.31%; Observed C:62.87, H:5.14,N:6.28, S:7.19%.

Synthesis of 8,8-dimethyl-BTD (10)

L-Penicillamine (410mg) was dissolved in 0.5N-HCl and added to a solution of compound (5, 700mg) in EtOH (10ml). NaOAc (310mg) was added to the mixture. The reaction mixture was stirred at room temp. for 20 hr. In this case no turbidity was observed during the reaction. Evaporation of the solvent afforded 650 mg of the residue, which could not be purified in spite of various trials. Then, the residue was subjected to the next step without purification.

The thiazolidine compound was dissolved in DMF (15ml) and heated under N_2-atmosphere in an oil-bath of 90° for 26hr. Evaporation of the reaction mixture afforded solid mass coloured light brown. After various unsuccessful trials of purification, chromatography with silica gel (solvent $CHCl_3$: MeOH: AcOH=96:3:1) afforded a small amount of crystals (22mg) in pure form. Although the other fractions was found to contain the same compound by tlc, it could not be isolated. M.p. 295° (dec), $[\alpha]_D$ -210° (c 0.5, DMF, t=23°), tlc Rf=0.25 ($CHCl_3$: MeOH: AcOH = 91:8:1). ms (EI) m/e=374. Elem anal. $C_{18}H_{18}N_2O_5S \cdot 2.5H_2O$ requires C:53.68, H:49.9, N:7.30, S:8.36%; Observed C:56.52, 4.65, N:7.15, S:7.59%.

ENKEPHALIN analogue

Synthesis of Boc-Phe-Leu-NH_2 (14)

Triethylamine (139μl) and DMF (10ml) were added to Boc-L-phenylalanine (265mg). The mixture was cooled in an ice-bath, and Leu-NH_2 (167mg), N-hydroxysuccinimide (115mg) and WSCD.HCl were added in the order under stirring and ice-cooling. After stirring at room temp. overnight, the reaction mixture was evaporated *in vacuo*. The residue was dissolved in a mixture of $CHCl_3$ and CH_2Cl_2 (1:1), and successively washed with water, 10% citric acid, satd. NaCl and 5% $KHCO_3$. After drying over Na_2SO_4, evaporation of the solvent, addtion of MeOH to the residue and re-evaporation afforded crystals of the product (246mg, 65% yield). Recrysatallization of the raw product from hot EtOH yielded (164mg, 43% yield). m.p. 174-176° [α]$_D$=-20.7° (c 0.35, DMF, t=25°).

Synthesis of N-phthalyl-BTD-Phe-Leu-NH_2 (15)

N-Phthalyl-BTD (7, 104mg) and H-Phe-Leu-NH_2 (94mg) were placed in a flask. A solution of NEt_3 (140μl) in DMF (7.5ml) was added. Then, N-hydroxysuccinimide (35mg) was added and cooled in an ice-bath. After addition of WSCD.HCl (58mg) under stirring, the reaction mixture was stirred at room temp. overnight. After evaporation of the reaction mixture the residue was dissolved in EtOAc and the solution was washed successively with 5%-$KHCO_3$, water, 10% citric acid and satd. NaCl, and dried over Na_2SO_4. Evaporation of the filtered solution afforded oily residue (145mg), which was then dissolved in CH_2Cl_2 (1.0ml), and precipitated by addition of Et_2O (5ml) and leaving overnight in a refrigerator. The ppt. formed was filtered and dried *in vacuo*. m.p. 222-226°, [α]$_D$= -53.9° (c 1.16, $CHCl_3$, t=26°). Elem. Anal. $C_{31}H_{35}N_5O_6S$ requires C:61.47, H:5.82, N:11.56, S:5.29%; Observed C:61.11, H:5.66, N:11.45, S:5.13%.

Synthesis of Boc-[BTD$^{2-3}$Leu^5]enkephalin amide (16)

Compound (15, 90mg) was treated with hydrazine hydrate (180μl) in DMF (1.0ml) at room temp. overnight. The crude product (15mg) was obtained by filtration of the reaction mixture, and it was used for next step without purification. It showed m.p. 228-238° and [α]$_D$ -128° (c 0.34, DMF, t=24°). It was dissolved in DMF (3.5ml) and Boc-Tyr-OSu (15mg) and a solution of NEt_3 (3.6mg) in DMF (3.5ml, a portion of previously prepared solution) was added thereto. The reaction mixture was stirred at room temp. for 18hr. Evaporation of the solvent afforded 32mg of the residue, which was purified by chromatography (silica, $CHCl_3$: MeOH = 6:1). Combination of the main-spot fractions and treatment with MeOH and Et_2O gave crystals (8mg). M.p. 145-146°; [α]$_D$ -68.0° (c 0.51, MeOH, t=22°); tlc Rf=0.55 (silica, $CHCl_3$: MeOH = 5:1). Elem. Anal. $C_{31}H_{35}N_5O_6S$ requires C:61.47, H:5.82, N:11.84, S:5.29%; Observed C:61.11, H:5.66, N:11.45, S:5.13%.

Synthesis of [BTD$^{2-3}$,Leu^5]enkephalin amide (17)

The Boc-protected compound (16, 14mg) was dissolved in formic acid (3ml) and treated with 2N-HCl in EtOAc (150μl) for 90min. at room temp. The reaction mixture was evaporated, and then addition of MeOH (2ml) and evaporation were repeated twice for complete removal of formic acid. The residue (14mg) was purified by chromatography (LH-20, MeOH) and ninhydrin positive fractions were collected, combined and evaporated to afford the product (9.5mg). M.p. 248-258° (dec.), [α]$_D$ -60.0° (c 0.5, MeOH, t=24°). FABMS m/e: 639(M+1), 481, 346, 318, 226, 199, 136. Amino Acid Anal. Leu:1.13, Tyr:0.93, Phe:1.00, NH_3:1.38, Cys:0.20, Pro:0.53 (BTD itself gave Cys:0.16, Pro:0.33). Elem. Anal. $C_{32}H_{42}N_6O_6S\cdot 2.5\ H_2O$ requires C:53.36, H:6.71, N:11.66%, Observed C:53.50, H:6.52, N:11.46%.

Bioassay of the enkephalin analogue

The longitudinal muscle strip of guinea pig ileum was suspended in an organ-bath and perfused with oxygenated Krebs-Ringer solution of 30° . The muscle strip was stimulated transmurally at 0.05Hz with pulse duration of 1ms. The contractile responses of the muscle strip were recorded isotonically, and the depression of the responses by addition of the opioid peptide was measured.

LRF analogue

Synthesis of Boc-BTD-Arg(NO_2)-Pro-Gly-NH_2 (22)

H-Arg(NO_2)-Pro-Gly-NH_2.HBr (21, 470mg) prepared by Fujino's method[19] was dissolved in DMF (2.0ml), and dioxane (2.0ml) and NEt_3 (98μl) were added. The mixture was treated with Boc-BTD-OSu in dioxane which was prepared from Boc-BTD-OH (9, 253mg), hydroxysuccinimide (101mg) and DCCI (182mg) in dioxane and EtOAc (1:1, 4.0ml). After 4hr. reaction at room temp. the reaction mixture was filtered to remove some insoluble and evaporated. The residue was distributed between n-BuOH and water, and the BuOH layer was evaporated after drying gave oily residue, which was triturated with Et_2O to give crystalline product (402mg, 86% yield). The crude product was then purified by chrmatography with LH-20 (MeOH), and with silica gel ($CHCl_3$: MeOH = 5:1). tlc Rf=0.19 ($CHCl_3$: MeOH = 5:1); Rf=0.40 (BuOH: AcOH: H_2O = 4:1:1). M.p. 155-160°(foam); [α]$_D$ -142.7°(c 1.0, MeOH, t=23°). Amino Acid Anal., Arg: Pro: Gly= 0.98: 1.11: 1.00 (=standard).

Synthesis of Boc-Ser-Tyr-$NHNH_2$ (19)

Boc-Ser-Tyr-OEt (1.24g) prepared starting from Boc-Ser(Bzl)-OH and H-Tyr-OEt.HCl were dissolved in DMF (15ml) and treated with hydrazine hydrate (3.03ml) overnight at room temp. Filtration and evaporation of the reaction mixtue afforded oily residue, which was crystallized by treatment with MeOH and Et_2O. Yield 975mg (82% yield), m.p. 196-199° , [α]$_D$ -22.0° (c 0.5, MeOH, t=24°). Elem. Anal., $C_{17}H_{26}N_4O_6\cdot 2.5H_2O$

requires C:52.16, H:6.95, N:14.31%; Observed C:51.98, H:7.02, N:14.10%.

Synthesis of Boc-Ser-Tyr-BTD-Arg(NO_2)-Pro-Gly-NH_2 (23)

H-BTD-Arg(NO_2)-Pro-Gly-NH_2 was prepared by treatment of compound (22) with 0.1N HCl in formic acid. This compound (364mg) was dissolved in DMF (2.0ml) and neutalized with NEt_3 (21μl), and then treated with the azide that was prepared by treating compound (26, 57mg) in DMF (2.0ml) with 2N HCl/EtOAc (0.3ml), isoamylonitrile (23ul) and NEt_3 (84ul) by the usual procedure. The reaction was continued until the spots of starting materials almost disappeared. The reaction mixture was filtered and evaporated, and the residue was distributed between n-BuOH and water. The organic layer was washed with water, 10% citric acid twice and water, and then evaporated to dryness *in vacuo*. The residue was crystallized by treating with MeOH and Et_2O. The crude product was purified by chromatography with LH-20 (in MeOH). Yield 87mg (63% yield), m.p. 160-165° (foam), [α]$_D$ -123.7° (c 1.0, MeOH, t=24°). Amino Acid Anal., Ser: Tyr: Arg: Pro: Gly= 0.99: 0.99: 0.99: 1.03: 1.00(=standard).

Synthesis of pGlu-His-Trp-Ser-Tyr-BTD-Arg(NO_2)-Pro-Gly-NH_2 (24)

H-Ser-Tyr-BTD-Arg(NO_2)-Pro-Gly-NH_2.HCl was prepared by treatment of compound (23) with 2N HCl/EtOAc in formic acid. This compound (86mg) was dissolved in DMF (2.0ml), and NEt_3 (14μl), compound 18 (45mg) and HONb (27mg) were added, and finally under ice cooling DCCI (31mg) was added. Stirring was continued for 20hr, during which the temperature was allowed to rise to room temp. spontaneously. The reaction mixture was filtered and evaporated, the residue was dissolved in MeOH (3.5ml) and DMF (0.5ml), and then precipitated by addition of Et_2O (8ml), and kept in a refrigerator overnight. The ppt. were filtered and dried to give 125mg of the product, which was then purified by chromatography with LH-20 (in DMF). Evaporation of combined main-spot fractions and trituration with a mixture of CH_2Cl_2 and MeOH (1:1) afforded the powder of desired product (71mg, 56% yield). M.p. 191-5°, [α]$_D$ -79° (c 1.0, DMF, t=24°). Amino Acid Anal., Ser: Tyr: Arg: Pro: Gly= 0.99: 0.99: 0.98: 1.10: 1.00(=standard).

Syntheis of [BTD$^{6-7}$]LRF (25)

Compound (24, 31mg) was dissolved in HF/anisole (90:10, v/v), and kept at 0° for 60min. After evaporation of volatile substances the residue was washed twice with Et_2O to remove anisole, dissolved in 25% AcOH and lyophilized. The crude product was purified by HPLC (ODS, 30% acetonitrile/water containing 0.1% TFA). The main peak fractions were collected and evaporated, then dissolved in 25% AcOH and lyophilyzed. Final yield was 9.8mg. FAB/MS: M^+ 1209 (MW= 1210.33). [α]$_D$ -58.2° (c 0.5, 5% AcOH, t=24°). uv (5% AcOH) λ max 277nm (ε 5810). Amino Acid Anal. (acid hydrolysis) Glu: His: Ser: Tyr: Arg: Pro: Gly= 1.02: 1.02: 1.01: 1.01: 1.01: 0.93:

1.00(=standard).

Bioassay of compound (25)

The biological activity of compound (25) was assayed using two systems a) *in vitro* assay: Pituitary of juvenile female rats was isolated and dispersed by treatment with collagenase. After washing several times the pituitary fragments were sunspend in Herpes buffer, transferred to the tissue culture dishes and cultured 5 days. After removal of the medium, the LH released 4hr after addtion of the peptide was measured by RIA[23]. b) *in vivo* assay: Blood LH was measured by RIA 15 min. after caudal vein injection of native LRF or compound (25) to young male rats. Three rats of average body weight 250g was used for each dose (4 doses were used).

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