Synthetic and Biological Activity Studies on a New Cyclic Pentapeptide, Cyclonitroproctolin

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The synthesis of a new biologically active cyclic pentapeptide, cyclonitroproctolin, cyclo(-Arg (NO_2)-Tyr-Leu-Pro-Thr-) by solution phase synthesis is described. The structure of the synthetic peptide was characterized by IR, NMR, FAB mass and analytical data. The newly synthesized compound was screened for its antimicrobial and pharmacological activities.

Key words: Cyclonitroproctolin, Antimicrobial Activity, Pharmacological Activity, *p*-Nitrophenyl Ester Method

Introduction

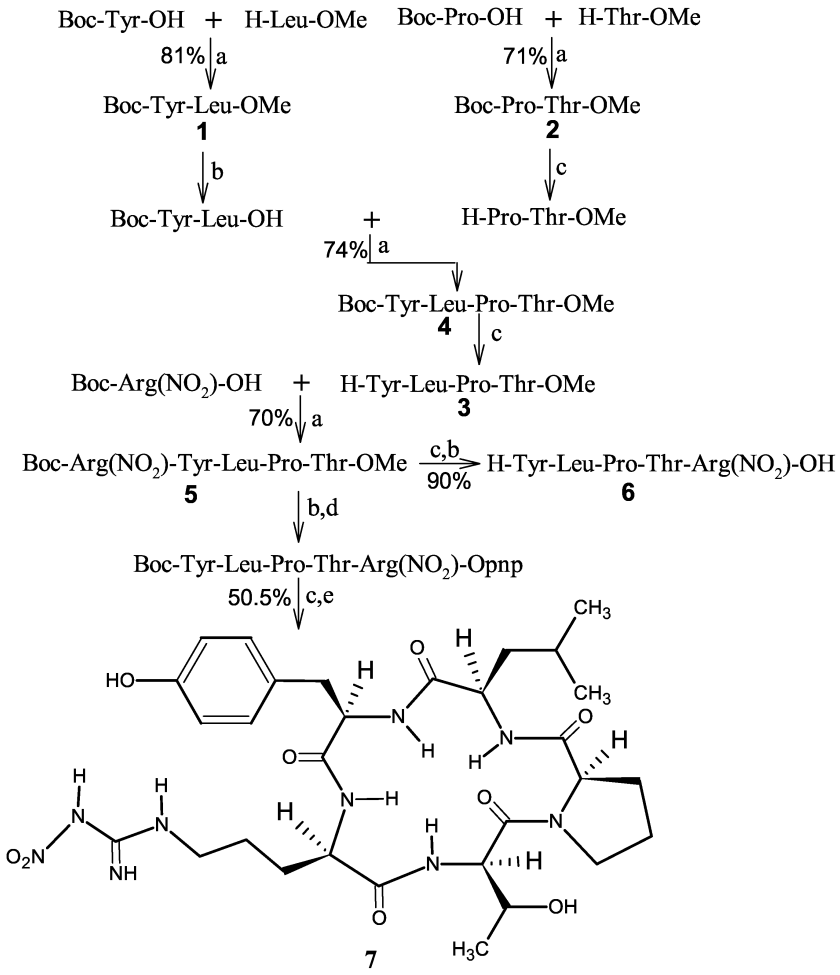
It is reported in the literature that a wide variety of naturally occurring bioactive cyclic peptides have been isolated from plants, marine sponges and tunicates [1] in the past two decades. Recently a large number of these cyclic peptides have been emerging as an important class of organic compounds due to their unique structures and biological activities. The wide spread increase of bacterial resistance towards conventional antibiotics encourages the exploration of novel antimicrobial molecules with unexploited mechanisms. Initially discovered as a defensive system in invertebrates and vertebrates, antimicrobial peptides are attracting increased interest as potential therapeutics [2 – 4]. Unlike classical antibiotics, which must penetrate the target cell, the principle mode of action of peptides involves perturbation and permeabilization of the cell membrane. This mechanism confers activity towards a broad spectrum of microbial cells, but is also responsible for undesired lytic activity against mammalian cells such as erythrocytes [5 – 7].

In 1975, Brown and his group reported the isolation of a myotropic substance from the cockroach, *Perplacenta americana* which was identified as H-Arg-Tyr-Leu-Pro-Thr-OH [8, 9]. The synthesis of proctolin was also undertaken by Brown *et al.* [10]. Further studies on this compound have shown that proctolin functions as an excitatory neuromuscular transmitter in insect visceral muscle [11, 12].

In continuation of our research work of synthesizing cyclic peptides of biological interest [13], it was contemplated to synthesize nitroproctolin, H-Arg(NO_2)-Tyr-Leu-Pro-Thr-OH (**6**) and then cyclize it to yield cyclonitroproctolin (**7**) using the *p*-nitrophenyl ester method. The synthesized product was further evaluated for antimicrobial, antiinflammatory and anthelmintic activities.

Results and Discussion

In order to carry out the total synthesis of cyclonitroproctolin, two dipeptide units Boc-Tyr-Leu-OMe (**1**) and Boc-Pro-Thr-OMe (**2**) were prepared by coupling corresponding Boc-L-amino acids with the respective L-amino acid methyl ester hydrochlorides using DCC, HOBt and *N*-methyl morpholine according to Bodanszky procedure [16] with suitable modifications [14]. The required Boc-amino acids [14] and amino acid ester hydrochlorides [15] were prepared by the reported procedures. The protected amino acid unit, Boc-Arg(NO_2)-OH (**3**) was also prepared [17]. The ester group of the dipeptide **1** was removed with LiOH and the Boc-group of the dipeptide **2** was removed with trifluoro acetic acid (TFA). Both the deprotected units were coupled to obtain Boc-Tyr-Leu-Pro-Thr-OMe (**4**), which was then coupled with Boc-Arg(NO_2)-OH (**3**) after appropriate deprotection to obtain Boc-Arg(NO_2)-Tyr-Leu-Pro-Thr-OMe (**5**). The guanidino group of arginine was protected by nitration to prevent the side reaction. Finally, cyclization of lin-



Scheme 1. a = DCC, NMM, HOBt, DCM, RT/36 h; b = LiOH, THF:H_2O(1:1), RT/1 h; c = TFA, $CHCl_3$, RT/1 h; d = *p*-nitrophenol (pnp), DCC, $CHCl_3$, RT/12 h; e = pyridine, $CHCl_3$, 10 days/0 °C.

ear pentapeptide **5** was carried out by the *p*-nitrophenyl ester method [18] to obtain cyclonitroproctolin **7** as depicted in Scheme 1.

The Boc and methyl ester protecting groups of **5** were also removed to yield nitroproctolin (**6**). The intermediates and final products were purified by column chromatography using a dichloromethane-methanol system and recrystallized from EtOAc-*n*-hexane.

The structures assigned to the intermediates and the final products are in good agreement with elemental analyses, IR, ^{1}H NMR and mass spectral data. The characteristic IR absorption bands of –CO-NH- and $–NO_2$ moiety were present in the cyclized product. ^{1}H NMR spectrum of the cyclized product clearly indicated the presence of all respective amino acid moieties. The absence of peaks corresponding to the *p*-nitrophenyl ester group in the IR, ^{1}H NMR and mass spectra of the cyclic peptide clearly confirmed the cyclization of linear segment. Characteristic IR absorption band of nitro group showed the presence of nitro-protected arginine intact in the title compound. The FAB mass spectrum showed the $(M^+ + 1)$ peak at *m/z* 676, which is consistent with the molecular formula, $C_{30}H_{45}N_9O_9$.

Biological Activity Studies

Cyclonitroproctolin was screened for its antibacterial, antifungal, anti-inflammatory and anthelmintic activity. The antibacterial and antifungal activity were carried out against four bacterial (*S. aureus*, *B. subtilis*, *P. aeruginosa* and *E. coli*) and two fungal strains(*C. albicans* and *A. niger*). These activity studies were carried out by the disc diffusion method [18]. Penicillin and griseofulvin were used as standards against bacteria and fungal strains at 50 μg/disc respectively. To

Table 1. Antibacterial and antifungal activity data of nitroproctolin and cyclonitroproctolin.

Compound	Diameter of zone of inhibition					
	Antibacterial activity data			Antifungal activity data		
	P.aer	*E.coli*	*B.sub*	*S.aur*	*C.alb*	*A.niger*
Nitroproctolin (**6**)	14	12	18	12	18	12
Cyclonitroproctolin (**7**)	20	14	20	16	20	18
Penicillin	12	12	18	18	–	–
Griseofulvin	–	–	–	–	20	20

Table 2. Antiinflammatory activity data of cyclonitroproctolin.

Compound	Increase in paw volume after 3 h ± S E [ml]	Percentage inhibition of oedema after 3 h ± S E
Cyclonitroproctolin (**7**)	0.55 ± 0.03	39.56
Ibuprofen	0.55 ± 0.04	40.50
Control	0.91 ± 0.02	–

Table 3. Anthelmintic activity data of cyclonitroproctolin.

Compound	Conc. of the Compd (mg)	Mean paralyzing time(min) ± S E	Mean death time (min) ± S E
Cyclonitro-	100	65.30 ± 2.01	99.24 ± 2.03
proctolin (**7**)	200	58.60 ± 2.05	89.94 ± 2.15
Mebendazole	100	17.44 ± 0.09	54.40 ± 1.08
	200	12.14 ± 1.02	31.04 ± 1.09
Control	–	–	–

compare and contrast, the antibacterial and antifungal activity of linear and cyclic nitroproctolin was carried out at 50 μg/disc. The results summarized in Table 1 indicate that the cyclized product shows good activity in comparison to the linear segment. The results also indicate that compound shows moderate to excellent activity against the bacterial and fungal strains tested as compared with the standards used. The anti-inflammatory activity was carried out according to the method of Winter *et al*. [19] using Ibuprofen as the standard and the results are presented in Table 2. The anti-inflammatory data reveal that the compound possesses good anti-inflammatory activity in comparison with the standard test compound. The anthelmintic activity was carried out against the earthworms (*Pontoscolex corethruses*) according to Garg's method [20] using Mebendazole as standard drug (Table 3). The compound is found to be moderately active as compared to the standard.

Conclusion

In conclusion, a new biologically active cyclic pentapeptide, cyclonitroproctolin, was successfully synthesized by solution phase method. The antimicrobial results indicated the better activity of synthetic cyclic peptide than its linear analogue and it proved to be a good antimicrobial agent. Evaluation of antiinflammatory activity of it revealed good activity as compared to the standard drug, Ibuprofen.

Experimental Section

Melting points were taken in open capillary and are uncorrected. IR spectra (in $CHCl_3$) were recorded on a Perkin-Elmer infrared spectrophotometer. NMR spectra were recorded in $CDCl_3$/DMSO-d_6 on a 300 MHz spectrophotometer using TMS as an internal standard. The mass spectra were recorded on a FAB mass spectrometer. The progresses of the reactions were checked by TLC on silica gel G plates and the products were purified by silica gel column chromatography.

For the protection of amino group of amino acids, ditertiary butyl pyrocarbonate(Boc-O-Boc) was used. The carboxy group of amino acids was protected by esterification. Bodanszky procedure with modification was used for the synthesis of peptides. Boc-group was removed by stirring the Boc-amino acid/peptide (1 mmol) with CF_3CO_2H (2 mmol) in $CHCl_3$ (15 ml) for 1 h at r. t. and the ester group was removed by stirring the amino acid/peptide methyl ester (1 mmol) with LiOH (1.5 mmol) in (1:1) THF:H_2O (16 ml) for 1 h at r. t. The *p*-nitrophenyl ester method was employed for cyclization of the linear segment **5**.

*Boc-tyrosinyl-leucine methyl ester (**1**)*

IR($CHCl_3$): $\nu = 3500$ (br.s, O-H str.), 3200 (br.s, N-H str.), 3050 (m,=C-H str.), 2980 (m, C-H str.), 2820 (m, C-H str.),1710 (s, C=O str. ester), 1670 (s, C=O str. amide), 1610 (m, C=C str.), 1445 (s, C-H def.), 1420 (m, C-N str.), 1395 (s, N-H def.), 1200 (s, C-O str.), 1150 (m, C-O str.), 915 – 850 (m, C-H def.) cm^{-1}. – *^1H NMR* (300 MHz, $CDCl_3$): $\delta = 8.9$ (br.s, 1H, -NH), 7.0 (d, 2H, $J = 6.5$ Hz, Ar-C_8H & -C_5H), 6.85 (d, 2H, $J = 6.5$ Hz, Ar-C_2H & -C_6H), 6.4 (br.s, 1H, -NH), 6.1 (br.s, 1H, -NH), 4.9 – 4.7 (m, 1H, α-CH), 4.5 – 4.4 (m,1H, α-CH), 3.75 (s, 3H, -OCH_3), 3.1 – 3.0 (m, 2H, β-CH_2), 1.6 – 1.5 (m, 2H, -CH_2), 1.4 (s, 9H, ^{t}Boc), 1.2 – 1.1 (m, 1H, -CH-), 0.95 (d, 6H, $J = 6.5$ Hz, -CH_3). – $C_{21}H_{32}N_2O_6$ (330): calcd. C 61.76, H 7.84, N 6.86; found: C 61.82, H 7.90, N 6.84.

*Boc-prolyl-threonine methyl ester (**2**)*

IR($CHCl_3$): $\nu = 3400$ (br.s, O-H / N-H str.), 2950 (m, C-H str.), 2890 (m, C-H str.), 1720 (s, C=O str. ester), 1665 (s, C=O str. amide), 1610 (m, C=C str.), 1450 (s, C-H def.), 1420 (m, C-N str.), 1405 (s, N-H def.), 1150 (s, C-O str.), 1100 (m, C-O str.) cm^{-1}. – *^1H NMR* (300 MHz, $CDCl_3$):

$\delta = 6.1$ (br.s, 1H, -NH), 5.15 (s, 1H, -OH), 4.9 – 4.4 (m, 2H, β-CH & α-CH), 4.3 – 4.1 (m,1H, α-CH), 3.7(s, 3H, -OCH_3), 3.6 – 3.2 (m, 2H, *N*-CH_2), 2.3 – 1.6 (m, 4H, -CH_2- CH_2), 1.4 (s, 9H, ^{t}Boc), 1.1 (d, 3H, $J = 6.5$ Hz, CH_3). -$C_{15}H_{26}N_2O_6$ (330): calcd. C 54.54, H 7.87, N 8.48; found C 54.50, H 7.85, N 8.44.

*Boc-tyrosinyl-leucyl-prolyl-threonine methyl ester (**4**)*

IR($CHCl_3$): $\nu = 3600$ (br.s, O-H str.), 3200 (br.s, N-H str.), 3080 (m, =C-H str.), 2920 (m, C-H str.), 2880 (m, C-H str.), 1740 (s, C=O str. ester), 1690 (s, C=O str. amide), 1670 (s, C=O str. amide), 1450 (s, C-H def.), 1430 (m, C-N str.), 1390 (s, N-H def.), 1300 (s, C-O str.), 1250 (m, C-O str.), 950 – 850 (m, C-H def.) cm^{-1}. – *^{1}H NMR* (300 MHz, $CDCl_3$): $\delta = 8.2$ (br.s, 2H, -NH), 7.6 (br.s, 1H, -NH), 7.2 (d, 2H, $J = 6.5$ Hz, Ar-C_8H & -C_5H), 6.95 (d, 2H, $J = 6.5$ Hz, Ar-C_2H & -C_6H), 6.5 (br.s, 1H, -NH), 5.2 (s, 1H, -OH), 4.9 – 4.8 (m, 1H, β-CH), 4.65 – 4.4 (m, 3H, α-CH), 4.3 – 4.0 (m, 2H, α-CH), 3.75 (s, 3H, -OCH_3), 3.6 – 3.2 (m, 4H, *N*-CH_2 & β-CH_2), 2.2 – 1.6 (m, 4H, -CH_2- CH_2), 1.6 – 1.5 (m, 1H, β-CH_2), 1.4 (s, 9H, ^{t}Boc), 1.3 (d, 3H, $J = 6.5$ Hz, -CH_3), 1.2 – 1.1 (m, 1H, -CH-), 0.98 (d, 6H, $J = 6.5$ Hz, -CH_3). -$C_{30}H_{46}N_4O_9$ (606): calcd. C 59.40, H 7.59, N 9.24; found C 59.40, H 7.59, N 9.24.

*Boc-nitroargininyl-tyrosinyl-leucyl-prolyl-threonine methyl ester (**5**)*

IR($CHCl_3$): $\nu = 3600$ (br.s, O-H str.), 3320 (br.s, N-H str.), 3030 (m, =C-H str.), 2930 (m, C-H str.), 2880 (m, C-H str.), 1750 (s, C=O str. ester), 1690 (br.s, C=O str. amide), 1670 (s, C=O str. amide), 1530 (s, NO_2), 1450 (s, C-H def.), 1420 (m, C-N str.), 1400 (s, N-H def.), 1380 (s, NO_2), 1250 (s, C-O str.), 1110 (m, C-O str.), 970-870 (m, C-H def.) cm^{-1}. – *^{1}H NMR* (300 MHz, $CDCl_3$): $\delta = 8.3$ (br.s, 2H, -NH), 7.6 (br.s, 2H, -NH), 7.1 (d, 2H, $J = 6.5$ Hz, Ar-C_8H & -C_5H), 6.8 (d, 2H, $J = 6.5$ Hz, Ar-C_2H & -C_6H), 6.4 (br.s, 3H, -NH), 5.2 (s, 1H, -OH), 4.9 – 4.7 (m, 4H, α-CH & β-CH), 4.6 – 4.4 (m, 3H, α-CH), 3.37 (s, 3H, O-CH_3), 3.5 – 3.2 (m, 6H, *N*-CH_2 & β-CH_2), 2.2 – 1.8 (m, 4H, -CH_2- CH_2), 1.65 – 1.5 (m, 6H, *N*-CH_2 & β-CH_2), 1.45 (s, 9H, ^{t}Boc), 1.3 (d, 3H, $J = 6.5$ Hz, -CH_3), 1.2 – 1.1 (m, 1H, -CH), 0.98 (m, 9H, 3 -CH_3). – $C_{36}H_{57}N_9O_{12}$ (807): calcd. C 53.50, H 7.00, N 15.58; found C 53.56, H 7.10, N 15.63.

*General procedure for the synthesis of cyclonitroproctolin (**6**)*

To the solution of Boc-nitroargininyl-tyrosinyl-leucyl-prolyl-threonine-*p* -nitrophenyl ester (1.2 mmol) in chloroform (15 ml), trifluoroacetic acid (0.274 g, 2.4 mmol) was added, stirred for 1 h at r. t. and washed with 10% sodium bicarbonate solution. The organic layer was dried over anhydrous sodium sulphate. To the resulting Boc-deprotected peptide-*p*-nitrophenyl ester in tetrahydrofuran (15 ml), pyridine (1.4 ml, 2 mmol) was added and kept at 4 °C for ten days. The reaction mixture was washed with 10% sodium bicarbonate solution until the byproduct *p*-nitrophenol was removed completely and finally washed with 5% HCl (5 ml). The organic layer was dried over anhydrous sodium sulphate. Tetrahydrofuran and pyridine were distilled under reduced pressure to get cyclonitroproctolin. The crude product was purified by silica gel column chromatography using the dichloromethane-methanol system and finally recrystallized from EtOAc-*n*-hexane.

Pale white solid; m.p. 153 – 155 °C. – *IR*($CHCl_3$): $\nu = 3600$ (br.s, O-H str.), 3325 (br.s, N-H str.), 3020 (m, =C-H str.), 2920 (s, C-H str.), 2850 (s, C-H str.), 1690 (s, C=O str. amide), 1680 (s, C=O str. amide), 1660 (s, C=O str. amide), 1610 (s, C=N str.), 1540 (s, NO_2), 1450 (s, C-H def.), 1410 (m, C-N str.), 1400 (s, N-H def.), 1380 (s, NO_2), 1110 (m, C-O str.), 970 – 915 (m, C-H def.) cm^{-1}. – *^{1}H NMR* (300 MHz, DMSO-d_6): $\delta = 8.2$ (br.s, 2H, -NH / OH), 7.6 (br.s, 3H, -NH), 7.0 (d, 2H, $J = 6.5$ Hz, Ar-C_8H & -C_5H), 6.9 (d, 2H, $J = 6.5$ Hz, Ar-C_2H & -C_6H), 6.4 (br.s, 3H, -NH), 5.2 (s, 1H, -OH), 4.7 – 4.5 (m, 4H, α-CH & β-CH), 4.4 – 4.2 (m, 3H, α-CH), 3.5 – 3.1 (m, 4H, N-CH_2 & β-CH_2), 2.2 – 1.7 (m, 8H, -CH_2-CH_2 & -CH_2), 1.6 – 1.4 (m, 4H, β-CH_2 & -CH_2), 1.2 – 1.1 (m, 4H, γ-CH & -CH_3), 0.95 (d, 6H, $J = 6.5$ Hz, $C(CH_3)_2$). – *FAB mass*: *m/z* 676 [M^+ + H]. -$C_{30}H_{45}N_9O_9$ (675): calcd. C 53.30, H 6.60, N 18.65; found C 53.38, H 6.66, N 18.74.

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