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Insect neuropeptide antagonist. Part II. Synthesis and biological activity of backbone cyclic and precyclic PBAN antagonists

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Abstract: A new approach for the design and synthesis of pheromone biosynthesis activating neuropeptide (PBAN) agonists and antagonists using the backbone cyclization and cycloscan concepts is described. Two backbone cyclic (BBC) libraries were synthesized: library I (Ser library) was based on the active C-terminal hexapeptide sequence Tyr-Phe-Ser-Pro-Arg-Leu-NH_2 of PBAN1-33NH_2; whereas library II (D-Phe library) was based on the sequence of the PBAN lead linear antagonist Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2. In both libraries the Pro residue was replaced by the BBC building unit N^{α}-(ω-aminoalkyl) Gly having various lengths of alkyl chain. The peptides of the two libraries were tested for agonistic and antagonistic activity. Four precyclic peptides based on two of the BBC antagonists were also synthesized; their activity revealed that a negative charge at the N-terminus of the peptide abolished antagonistic activity. We also describe the use of the reagent $SiCl_3I$ for selective deprotection of the Boc group from the building unit prior to on-resin amino-end to backbone-nitrogen (AE-BN) cyclization, during solid-phase synthesis with Fmoc chemistry.

Abbreviations: ACN, acetonitrile; BBC, backbone cyclic; Boc, *t*-butoxycarbonyl; DCM, dichloromethane; DIEA, diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodimide; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexaflurophosphate; MBHA, methylbenzhydrylamine; NMP, N-methylpyrrlolidone; NMR, nuclear magnetic resonance; OBzl, benzyl ester; PBAN, pheromone biosynthesis activating neuropeptide; Pmc, 2,2,5,7,8-pentamethyl chroman-6-sulfonyl; PyBroP,

bromo-tris-pirrolidino-phosphonium hexafluorophosphate; RP-HPLC, reverse phase high-performance liquid chromatography; SAR, structure activity relationship; $SiCl_3I$, iodotrichlorosilane; SMPS, simultaneous multiple peptide synthesis; SPPS, solid-phase peptide synthesis; SPS, solid-phase synthesis; tBu, *t*-butyl; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TOF-MS, time of flight mass spectrometry. Standard IUPAC abbreviations for amino acids are used. The building units for backbone cyclization are abbreviated by the three-letter code of the corresponding modified amino acid, followed by the type of functional group at the end of the N-alkylene chain (N for amine, C for carboxyl, S for thiol) and an indication of the number of spacing methylene groups. For example, GlyN2 describes a modified Gly residue with a β-amino functional group and an N-ethyl (two methylene groups) spacer.

The cyclization of peptides is one of the commonest and most attractive methods of introducing conformational constraints into peptides and thus restricting their conformational space (1). The conformational constraints confer the following attributes on the peptides: (i) selectivity, (ii) enhanced metabolic stability, (iii) increased biological activity, and (iv) improved bio-availability. Natural modes of peptide cyclization (2) cannot easily be applied to most peptides and their application often causes a loss of or marked reduction in bioactivity. A method called 'backbone cyclization' (2) overcomes the above limitations. In this method ring closure is performed by attachment of the ω-functional groups namely amino, carboxy or thiol to the backbone nitrogen by an alkylene tether of changing length, whereas the side-chains and terminal functional groups, which may be essential for biological activity, are retained. Thus, the peptide bond, N-H is replaced with N-R-G-Pg (where R is an ω-functional alkylene moiety of various lengths, G is a functional group and Pg is an orthogonal protecting group). The modes of backbone cyclization are: (i) amino end to backbone nitrogen (AE-BN), (ii) side-chain to backbone nitrogen (SC-BN), (iii) backbone nitrogen to backbone nitrogen (BN-BN), and (iv) backbone nitrogen to carboxy end (BN-CE).

In order to obtain the optimal backbone cyclic (BBC) peptide based on a given sequence, namely the one that best fits the desired bioactive conformation, it is necessary to screen the conformational space of the linear peptide systematically. This is achieved using cycloscan (3). Cycloscan is defined as a selection method based on conformationally constraint BBC peptide libraries intended for the efficient screening of the conformational space and thus for fast discovery of a BBC peptide lead compound that overlaps the bioactive conformation.

Cycloscan is performed by designing and synthesizing libraries of BBC peptides and screening them with the appropriate bioassay. All the peptides in each library bear the same sequence, but differ from each other in distinct parameters which affect their conformation and hence their bioactivity. This is achieved by the introduction of gradual discrete conformational perturbations that allow efficient screening of the conformational space of the parent peptide. The majority of the peptides in such a library should be inactive, because they do not overlap the bioactive conformation, but the peptides that do fit the bioactive conformation should be highly potent and should have all the pharmacological advantages mentioned above.

The diversity of sequence-biased cycloscan is not sequential but conformational, and includes the following modes: (i) the modes of backbone cyclization, (ii) the position of the backbone bridge along the peptide sequence, (iii) the size of the bridge, (iv) the chemistry of the bridge. Each of these diversity parameters has been shown to affect the conformation and hence the biological activity of the peptide (4–6).

The concepts of backbone cyclization and cycloscan have been demonstrated in our laboratory with several biologically active peptides, such as substance P (2,4–6) and somatostatin (7). Extensive research on these peptides has clearly proved the feasibility and effectiveness of the concepts of BBC and cycloscan, and has made possible the use of these techniques on other peptides. In this study we applied this combined approach to the insect family of pyrokinin/PBAN neuropeptides in order to discover highly potent and selective antagonists.

PBAN is an important neuropeptide that mediates some of the key functions in insects. PBAN was first reported by Raina & Klun in 1984 (8), as the neuropeptide that regulates sex pheromone production in female moths, and its amino acid sequence was determined in 1989 for *Helicoverpa zea* (Hez-PBAN; PBAN1-33NH_2 (9). Since then, six other PBAN molecules have been isolated from five additional moth species by (10–15) and its cDNA and genes have been cloned (13–20). PBAN molecules were found to be C-terminally amidated neuropeptides consisting of 33–34 amino acid residues. All the molecules share a high degree of homology and an identical C-terminal pentapeptide sequence (Phe-Xxx-Pro-Arg-Leu-NH_2; Xxx=Ser) which also constitutes the active core required for their

biological activity (21–28). The common C-terminal sequence (Phe-Xxx-Pro-Arg-Leu-NH_2; Xxx=Ser, Gly, Thr, Val) is also shared by additional insect neuropeptides, all of which have recently been designated the pyrokinin/PBAN family: pyrokinins and myptropins (29,30), pheromonotropin (31) and diapause hormone (32). Studies of the biological activity of the pyrokinin/PBAN neuropeptides have revealed that, in addition to their ability to stimulate sex pheromone biosynthesis in moths, members of this family have been found to control melanization and reddish coloration in moth larvae (33,34), contraction of the locust oviduct (35), myotropic activity of the cockroach and locust hindgut (35,36), egg diapause in the silkworm (32) and acceleration of pupariation in fleshfly larvae (37).

Several reasons urged us to seek receptor-selective antagonists to PBAN: (i) the major role of PBAN and the pyrokinin/PBAN family in the physiology of moths and other insects, (ii) knowledge of the amino acid sequence of the neuropeptide and the information derived from its SAR studies, and (iii) the availability of biological assays which enable agonistic and antagonistic activities to be determined quantitatively (22,33,38).

In this study we describe the synthesis of BBC and precyclic analogs based on the C-terminal hexapeptide sequence derived from Hez-PBAN, Tyr-Phe-Ser-Pro-Arg-Leu-NH_2 (PBAN28-33NH_2) (which comprises the active core of the prokinin/PBAN molecules) (22–24,33) and on a potent linear antagonist of PBAN that was found in our previous study (39–41).

Experimental Procedures

Chemicals

Fmoc-protected amino acids with standard side-chain protecting groups, Rink amide MBHA resins and reagents for peptide synthesis were purchased from NOVA Biochem (Switzerland). Ultrapure quality solvents were purchased from Baker (USA). Other reagents were purchased from Aldrich. Building units for BBC peptides were prepared as previously described (42).

Synthesis of BBC peptide libraries I and II

Both cyclic peptide libraries (Fig. 1): library I (Ser library) and library II (D-Phe library) were synthesized using the SMPS 'tea bags' methodology (43) on Rink amide MBHA resin (loading 0.55 mmol/g), by means of Fmoc chemistry.

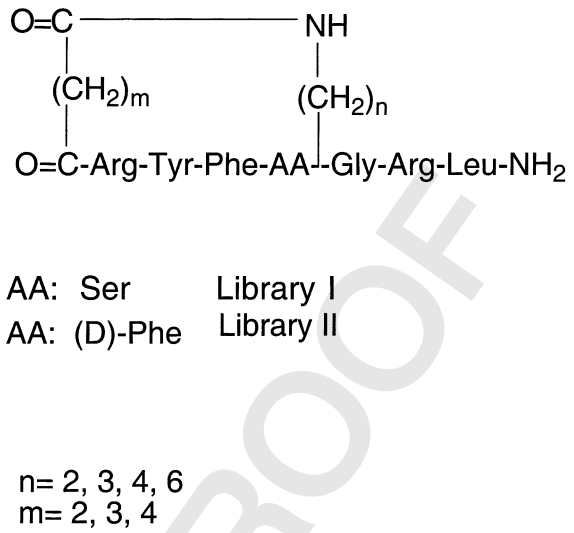


Figure 1. General structure of the BBC Ser and D-Phe peptide libraries. Incorporation of the building unit in the BBC peptide is depicted schematically by a line between the peptide bond (represented as a line between two amino acids) and the CH_2 group of the bridge.

The resin (150 mg) was placed in separate tagged and sealed polypropylene bags (5.5×5.5 cm), which were placed in polypropylene boxes. After swelling (NMP 3 h) and washing (NMP, 5×2 min; DCM 5×2 min and NMP 5×2 min), the Fmoc protecting group was removed with piperidine in NMP (20%, 2×30 min) and the resin was washed (NMP, 5×2 min; DCM 5×2 min and NMP 5×2 min). Coupling of protected amino acids was performed by pre-activation of a 3-fold excess of N^{α}-protected amino acid, a 3-fold excess of PyBroP and a 6-fold excess of DIEA in NMP for 10 min prior to coupling. After pre-activation, the activated amino acid was added to the bags and shaken vigorously (on a Big Bill shaker) for 2 h. The building units used were Fmoc-GlyN2, Fmoc-GlyN3, Fmoc-GlyN4 and Fmoc-GlyN6 [N^{α}-Fmoc, N^{α}-(Boc-NH-$(CH_2)_n$)Gly (n=2, 3, 4, 6)]. Couplings after the building units were repeated twice. The Fmoc group was deprotected as above.

Acylation with dicarboxylic acid

The last amino acid on the peptidyl-resin (Arg) was acylated with 10 eq. of succinic anhydride (m=2), or glutaric anhydride (m=3) or anhydride of adipic acid (m=4). Anhydride of adipic acid was prepared by reacting adipic acid DIPCDI in NMP for 20 min at room temperature. Acylation was performed by the appropriate anhydride in NMP, for 2 h at room temperature, in the presence of 1 eq. DMAP.

Selective Boc deprotection by $SiCl_3I$

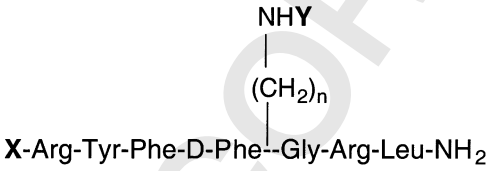
Deprotection of the Boc protecting group from the N^{ω}-(aminoalkyl) group of the building units was performed with $SiCl_3I$. $SiCl_3I$ was freshly prepared by stirring NaI (5.94 g, 39.6 mmol) and $SiCl_4$ (4.5 mL, 39.6 mmol) in 120 mL of DCM/ACN (1:1) at room temperature. Boc deprotection was carried out with $SiCl_3I$ (20-fold excess) in 240 mL of DCM/ACN (1:1) for 10 min at room temperature. The bags were washed twice with DCM/ACN (1:1) (2×250 mL), neutralized with 5% DIEA in NMP and washed again twice with NMP (2×250 mL).

On-resin cyclization

After Boc deprotection the peptides were cyclized by the addition of 6 eq. PyBoP and 12 eq. DIEA in NMP (repeated twice) and then with 3 eq. PyBoP and 6 eq. DIEA in NMP. The bags were washed with NMP (3×250 mL) and DCM (3×250 mL). The peptidyl-resin was dried *in vacuo*, yielding ≈ 230 mg of dry resin in every bag.

Synthesis of precyclic peptides

Four precyclic peptides: 20L-1, 20L-2, 28L-1 and 28L-2 (Fig. 2) were synthesized using the SMPS 'tea bags' methodology as described above. Synthesis was started from 120 mg of Rink amide MBHA resin (loading 0.55 mmol/g) in every bag. After coupling of the last amino acid (Arg), Fmoc was deprotected and peptides 20L-1 and 28L-1 were acetylated with acetic anhydride in DMF, in the presence of DIEA, for 30 min at room temperature. Peptides 20L-2 and 28L-2 were acylated with



Peptide	X	Y	n	m
20-L-1	Ac	Ac	2	
28-L-1	Ac	Ac	6	
20-L-2	OC-$(CH_2)_m$-COOH	H	2	3
28-L-2	OC-$(CH_2)_m$-COOH	H	6	2

Figure 2. General structure of the precyclic peptides.

succinic (m=2) or glutaric anhydride (m=3) in DMF, in the presence of DMAP, for 2 h at room temperature. The Boc protecting group was removed as described above and the peptides were washed and dried.

Final deprotection/cleavage of peptides from the resin

The peptides were deprotected and removed from the resin by 90% TFA with the addition of scavengers (H_2O/ethanedithiol/thioanisole, 2.5 : 2.5 : 5). The resin was removed by filtration and washed with TFA (3×5 mL), and the combined TFA filtrates were evaporated to dryness by a stream of nitrogen. The oily residue was triturated three times with cold ether to remove the scavengers, and the ether was removed by centrifugation. The dry crude peptide was dissolved in ACN/H_2O (1 : 1) and lyophilized.

Crude peptides were purified by RP-HPLC on 'LichrosorbR' C_{18} (LICHROCARTE 250-10 cat. 16817) using ACN/H_2O (+0.1% TFA). Purity of the peptides was assessed by analytical RP-HPLC and was found to be in the range 90–95%. Purified peptides were characterized by TOF-MS and amino acid analysis of hydrolizates. Analytical data of the peptides are shown in Tables 1–3.

Determination of pheromonotropic agonistic and antagonistic activities

Agonistic and antagonistic activities were determined in *Heliothis peltigera* females by means of the pheromonotropic bioassay as described previously (38,39). Agonistic activity was determined by injection of a dose of 1 nmol of each of the peptides for 2 h. Antagonistic activity was determined by injection of a dose of 1 nmol of each of the tested peptides, together with 0.5 pmol PBAN1-33NH_2. Glands were excised 2 h post-injection and their pheromone contents were determined by capillary gas chromatography as described previously (22).

Results and Discussion

The backbone cyclization and cycloscan concepts were applied to the design and synthesis of two BBC libraries based on the C-terminal active sequence of PBAN (22–24,33). In the first step, a library of linear peptides was designed and a linear lead antagonist Arg-Tyr-Phe-(D)-Phe-Pro-Arg-Leu-NH_2 was disclosed (41). The sequences of the parent agonist, as well as that of the lead antagonist, were used to design and synthesize the two BBC peptides

Table 1. **Analytical data of the Ser library**

Peptide no.[a]	*n*	*m*	MS found	MS calc'd	Amino acid analysis				
					Tyr	Phe	Ser	Arg	Leu
4	2	2	1022.60	1022.20	0.90	0.82	0.86	1.81	1
5	2	3	1037.10	1036.20	0.84	0.94	0.96	1.67	1
6	2	4	1051.20	1050.30	0.85	0.99	1.07	1.83	1
7	3	2	1037.20	1036.20	0.97	0.93	0.87	1.98	1
8	3	3	1051.20	1050.30	0.83	0.85	1.03	1.55	1
9	3	4	1064.80	1064.30	0.91	0.93	0.94	1.62	1
10	4	2	1050.90	1050.30	1.19	0.99	0.91	2.09	1
11	4	3	1065.10	1064.30	1.21	1.14	1.14	2.19	1
12	4	4	1078.10	1078.30	1.12	1.04	1.10	1.90	1
13	6	2	1078.85	1078.30	1.03	0.98	0.95	2.08	1
14	6	3	1092.40	1092.30	0.91	0.99	1.06	1.79	1
15	6	4	1106.10	1106.40	0.80	0.70	1.10	1.76	1

a. For peptide structure see Fig. 1.

Table 2. **Analytical data of the (D)-Phe library**

Peptide no.[a]	*n*	*m*	MS found	MS calc'd	Amino acid analysis			
					Tyr	Phe	Arg	Leu
19	2	2	1082.80	1082.30	0.88	1.83	1.77	1
20	2	3	1098.10	1096.30	0.81	1.98	1.76	1
21	2	4	1110.80	1110.40	0.83	1.92	1.72	1
22	3	2	1098.20	1096.30	0.85	1.88	1.93	1
23	3	3	1112.70	1110.40	0.95	1.94	1.88	1
24	3	4	1125.00	1124.40	0.92	1.93	1.84	1
25	4	2	1110.70	1110.40	1.00	2.37	2.38	1
26	4	3	1125.80	1124.40	0.77	1.62	1.57	1
27	4	4	1139.40	1138.40	0.87	1.84	1.73	1
28	6	2	1138.90	1138.40	0.84	1.90	1.74	1
29	6	3	1153.10	1152.40	0.96	2.02	2.09	1
30	6	4	1167.60	1166.40	0.94	1.97	1.91	1

a. For peptide structure see Fig. 1.

Table 3. **Analytical data of the precyclic peptides**

Peptide[a]	*n*	Yield (%)	MS found found	MS calc'd	Amino acid analysis			
					Tyr	Phe	Arg	Leu
20-L-1	2	30	1084.6	1084.3	0.99	2.00	1.99	1
20-L-2	2	53	1114.6	1114.3	1.05	2.09	2.20	1
28-L-1	6	49	1140.6	1140.4	0.94	1.80	2.06	1
28-L-2	6	53	1156.6	1156.4	0.97	2.09	2.09	1

a. For peptide structure see Fig. 2.

libraries (Fig. 1). The first library (Ser library) was based on the active C-terminal hexapeptide sequence (Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2) of PBAN1-33NH_2, which was found to comprise the PBAN active core. The second library (D-Phe library) was based on the sequence of the lead antagonist: Arg-Tyr-Phe-(D)-Phe-Pro-Arg-Leu-NH_2. In both libraries, the Pro residue was replaced with the building units Fmoc-GlyN2, Fmoc-GlyN3, Fmoc-GlyN4 and Fmoc-GlyN6 (Fig. 1). The position of cyclization in the peptide chain was determined by the close structural similarity between Pro and the building unit: both are N^{α}-alkylated amino acids. We assumed that the Pro plays a structural role in establishing the bioactive conformation (44). The Gly building unit maintains the N-alkylated character of Pro, but has a functional group that allows cyclization. The ω-amino group of the Gly building unit was connected to the N^{α}-terminal amino group by a dicarboxylic acid spacer (Fig. 1, $m=2, 3, 4$). All the cyclic peptides in each library had the same primary sequence and the same ring location. The members of each library differed from each other in their bridge size and the position of the amide bond along the bridge.

Cyclic peptides that include BBC peptides containing lactam bridges are usually synthesized by SPS methodology, by means of 'on-resin cyclization'. These peptides are usually synthesized either by Fmoc chemistry on TFA-sensitive resins using Allyl/Alloc for second-order (semipermanent) protection of side-chains involved in the formation of the lactam bridges, or by tBu type for third-order (permanent) protection. Alternatively, on HF-cleavable resins, Fmoc may be used for first-order (temporary) protection, Boc, for second-order protection and benzyl-type protecting groups for third-order protection. The use of Boc for second-order protection on TFA-cleavable resin requires selective deprotection. In this study we used $SiCl_3I$ for this purpose. This reagent has previously been used for N^{α}-Boc deprotection during the SPS of peptides by Boc chemistry (45).

The selective deprotection of Boc was achieved by $SiCl_3I$, which does not cleave the peptide from the Rink amide MBHA resin (46). In order to check the stability of the side-chain-protecting groups towards $SiCl_3I$, the Fmoc-Arg (Pmc)-OH, Fmoc-Ser (tBu)-OH and Fmoc-Tyr (tBu)-OH used in the synthesis were treated with 20-fold excess of $SiCl_3I$ (relative to the side-chain-protecting groups) under conditions similar to those used for Boc deprotection. Reactions were stopped at intervals of 5, 10, 15 and 30 min and 2.5 h; the reaction products were analyzed by RP-HPLC. The results are summarized in Table 4. The results indicate

Table 4. **Results of treatment of Fmoc-AA(X)-OH with $SiCl_3I$**

Reaction time	Fmoc Arg(Pmc) (%)[a]	Fmoc Ser(^{t}Bu) (%)[a]	Fmoc Tyr(^{t}Bu) (%)[a]	Fmoc Asp(OBzl) (%)[a]
5 min	87	12	0	59
10 min	87	1	0	54
15 min	80	0	0	51
30 min	73	0	0	50
2.5 h	56	0	0	47

a. Percentage of the starting material left after the treatment with $SiCl_3I$ reagent.

that the Pmc group of Arg is relatively stable under Boc deprotection conditions, whereas the tBu protecting groups of Ser and Tyr are not. The stability of β-benzyl ester of Asp to the $SiCl_3I$ reagent, under the conditions described above, was also tested as a model of peptidyl resin ester linkage. The results indicated that benzyl ester was partially cleaved (40–50%). Thus, under the conditions used for Boc deprotection, peptides can be partially cleaved from resins such as 'Wang Resin', in which the linkage is of a benzyl type.

Examination of the peptide yields from Ser and D-Phe sublibraries revealed that peptides 15 (from the Ser library) and 20, 21 and 30 (from the D-Phe library) gave low yields after cyclization (≤ 20%) (Table 5). Such low yields may be caused by two major factors: (i) side-reactions during the cyclization reaction, because of cyclizations involving Ser and Tyr which were used without side-chain protection, or (ii) low propensity of the linear precursors to undergo cyclization. We considered that the second reason applied in this study, because no cyclic analogs that involved cyclization through Ser and/or Tyr were detected in the crude mixture by MS analysis. Also, other peptides with the same amino acid sequence gave satisfactory yields. In light of the fact that peptide 20 (from the D-Phe library) showed very high antagonistic activity (see below) and gave low cyclization yields, we tried to improve the cyclization yield of peptide 20. We examined various conditions for cyclization on the resin of the precyclic peptide HOOC-$(CH_2)_3$CONH-Arg (Pmc)-Tyr-Phe-(D)-Phe-N_2Gly-Arg (Pmc)-Leu-Resin. We used different coupling reagents such as HBTU, TBTU and PyBroP, with ranges of equimolar excess and reaction time. The results (Table 6) clearly indicate that none of the reaction conditions significantly improved the cyclization yield of peptide 20 which remained at 10–20%. The highest yield (20%) was achieved when the cyclization was performed twice with a 6-fold excess of PyBroP. These findings confirm our hypothesis that the low cyclization yield of peptide 20 was due to its low propensity to undergo cyclization.

Table 5. **Yields of the Ser and D-Phe BBC peptides**

Peptide no.[a] (Ser library)	Yield (%)	Peptide no.[a] (D-Phe library)	Yield (%)
4	27	19	52
5	24	20	10
6	34	21	20
7	70	22	61
8	53	23	55
9	58	24	35
10	n.d.	25	44
11	n.d.	26	59
12	n.d.	27	31
13	n.d.	28	71
14	n.d.	29	23
15	14	30	17

a. For peptide structure see Fig. 1.; n.d., not determined.

Table 6. **Effect of different conditions on cyclization yield of the BBC peptide 20 (D-Phe library)**

No.	Coupling reagent	Excess	Number of cycles[a]	Yield (%)
1	TBTU	6 eq.	1	11
2	TBTU	6 eq.×2	2	10
3	HBTU	6 eq.	1	14
4	PyBroP	6 eq.	1	19
5	PyBroP	6 eq.×2	2	20
6	PyBroP	6 eq.×3	3	18
7	TBTU	[6 eq.×2]+[3 eq.×1]	3	12
8	TBTU	[6 eq.×2]+[3 eq.×2]	4	15
9	TBTU	[6 eq.×2]+[3 eq.×3]	5	14

a. Time of each cycle: 2 h.

The peptides from the Ser and D-Phe libraries were tested for their agonistic and antagonistic activities by means of a pheromone bioassay (38). Table 7 highlights the antagonistic and agonistic activities of all the peptides from both libraries. Under the conditions tested, peptides from the Ser library (4–15) did not show any significant antagonistic activity at 1 nmol. Peptides 14, 10 and 6 from this library showed agonistic activity, with peptide 14 being the most potent (exhibiting 75% activity at 1 nmol, compared with the activity of 1 nmol PBAN1-33NH_2). Peptides 10 and 6 exhibited only 45 and 33% agonistic activity, respectively. All three peptides were active at 1 nmol. Lower doses

Table 7. **Summary of agonistic and antagonistic activity of the Ser and D-Phe BBC peptides**

Peptide no.[a] (Ser lib.)	Agonistic activity (%)[b]	Antagonistic activity (%)[b]	Peptide no.[a] (D-Phe lib.)	Agonistic activity (%)[b]	Antagonistic activity (%)[b]
4	5	28	19	0	25
5	0	0	20	10	96
6	33	0	21	62	81
7	0	6	22	2	74
8	0	0	23	0	24
9	1	0	24	0	19
10	45	7	25	0	55
11	17	0	26	1	0
12	29	0	27	0	19
13	20	3	28	0	77
14	75	33	29	0	34
15	23	27	30	2	36

a. For peptide structures see Fig. 1.
b. Agonistic activity is expressed as the ratio (as percentage) between the sex pheromone content elicited by the injection of each of the cyclic peptides and PBAN1–33NH_2 (at 1 nmol). Antagonistic activity is expressed as 100 minus the ratio (as percentage) between the pheromone content in the gland elicited by the injection of PBAN1-33NH_2 in the presence and absence of each of the peptides. The amount of sex pheromone elicited by 0.5 pmol PBAN1-33NH_2 ranged from 93 to 113 ng pheromone/female and that by 1 nmol PBAN1-33NH_2 ranged from 93 to 198 ng pheromone/female. In all experiments pheromone content was monitored with at least 10 females for each of the tested peptides. n.t., not tested.

revealed very low activity at all tested concentrations (1, 10 and 100 pmol) except for peptide 14 which showed 21% activity at 100 pmol (compared with that of 100 pmol PBAN1-33NH_2). The activity of this peptide was similar to that exhibited by the linear peptides PBAN28-33NH_2 and [Arg^{27}]PBAN27-33NH_2 (41). Other BBC peptides of the Ser library did not exhibit any significant agonistic activity. Five of the tested peptides from the D-Phe library (peptide 20, 21, 22, 25 and 28) showed a significant inhibitory activity (at a concentration of 1 nmol). The inhibitory activity of these peptides ranged from 55% (peptide 25) to a maximum of 96% (peptide 20). Most of the D-Phe peptides were devoid of agonistic activity under the experimental conditions. The only peptide that exhibited significant pheromonotropic agonistic activity was peptide 21.

As indicated above, the primary structure of the D-Phe BBC peptides was based on the linear lead antagonists (H-Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2), which inhibited the pheromone biosynthesis elicited by 0.1–0.3 pmol of exogenously administered (injected) PBAN1-33NH_2 by 70–80% (41). Interestingly, examination of the antagonistic activities of the D-Phe BBC peptides revealed that not all were active, and that those that were active differed in their potency (Table 7). The differences in activity among the members of the D-Phe library of BBC peptides may result either from structural changes or from differences in their conformation, arising from the constraint that the cyclization imposes on the compounds in the library. Because all the peptides in the D-Phe library had the same primary structure and bridge location, and differed only in bridge size and chemistry, it is most likely that the last two factors affected the activity most. In order to further examine the effect of these factors on bioactivity, we synthesized four precyclic linear peptides (based on the active D-Phe BBC peptides 20 and 28, Fig. 2) in which the building unit consisted of $(CH_2)_n$ side-chains (n=2 and 6, respectively) and a spacer $(CH_2)_m$ (m=3 and 2, respectively) (see Fig. 2) and tested their pheromonotropic agonistic and antagonistic activities.

As can be seen in Table 8, peptides 20L-1 and 28L-1 were antagonistic and did not exhibit agonistic activity. The activity of these peptides was similar to that of the linear lead antagonistic parent molecule (H-Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2) and to that of their respective BBC peptides 20, 28. Despite the similarity in their general patterns of activity, the two precyclic peptides exhibited differences in their inhibitory potency, and the one with the longer side-chain (peptide 28L-1) was less active than peptide 20L-1 (56 and 91% inhibition, respectively).

Table 8. **Summary of agonistic and antagonistic activity of the precyclic peptides**

Peptide[a]	Agonistic activity (%)	Antagonistic activity (%)
20L-1	0	91
20L-2	0	20
28L-1	0	56
28L-2	0	37

Agonistic and antagonistic activities were determined as described in the legend to Table 7.
a. For structure see Fig. 2.

Interestingly, this trend in activity was similar to that of the equivalent BBC peptides 20 and 28 that inhibited pheromone biosynthesis by 90 and 77%, respectively, hinting at the possibility that in the case of these two peptides the length of the side-chain of the building unit had an important effect on the biological activity. Another interesting aspect is related to the effect of the residues at the α- and ε-amino groups of the molecule. Comparison of the activity of 20L-1 with that of 20L-2 and of that of 28L-1 with that of 28L-2 revealed that the presence of the carboxylic group on the N-terminal end and a free amino group on the side-chain resulted in a marked decrease in antagonistic activity (Table 8), i.e. the presence of a negatively charged residue at the N-terminus of the peptide abolished the antagonistic activity. The above results were in accordance with findings obtained previously in our laboratory, on the effects of the introduction of acidic groups in the N-terminal part of the highly active and NK-1 selective analog Ac-Arg-Septide, in which the substitution of Asp for Arg resulted in an inactive peptide (Gilon *et al*., unpublished results). Based on the above results, we considered that the differences between the activities of the BBC peptides and those of their linear precyclic analogs indeed resulted from both: structural and conformational changes.

In this study we synthesized BBC peptides based on the sequence of an insect neuropeptide and investigated their bioactivity. This approach, applied to PBAN, has led to the discovery of several antagonists and agonists, which exhibited pheromonotropic activity and effectively inhibited sex pheromone biosynthesis in female *H. peltigera* moths. The use of cyclization in the design of antagonists with enhanced selectivity and metabolic stability has hitherto been successfully applied only to vertebrate peptides such as oxytocin, glucagon, enkephalins and luteinizing hormone-releasing hormone. Here we present a new general approach that combines backbone cyclization and cycloscan for the generation of agonistic and/or antagonistic cyclic neurohormones (47,48). To the best of our knowledge this is the first report on the successful application of this approach to insect neuropeptides. The only cyclic peptide that has been generated for the pyrokinin/PBAN family to date is an end-to-end cyclic octapeptide derived from the C-terminal part of Lem-PK (49). Beyond the immediate benefits introduced by the cyclic peptides as selective antagonists, this approach also facilitates determination of the conformational requirements for pheromonotropic agonistic and antagonistic activities that can be assessed by NMR (3,50,51) and X-ray (52) methods. This information is of major importance, as it may serve as a basis for the design of improved (small, cost-effective and having enhanced metabolic stability and bioavailability), non-peptide, mimetic agonists and antagonists. Such compounds would be potential candidates for agrochemical applications and serve, after formulation and preliminary field experiments, as prototypes for the development of a novel group of highly effective, insect-specific and environment friendly insecticides.

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References

1. Kessler, H. (1982) Conformation and biological activity of cyclic peptides. *Angew Chem. Int. Ed. Engl.* **21**, 512–523.
2. Gilon, C., Halle, D., Chorev, M., Selinger, Z. & Byk, G. (1991) Backbone cyclization: a new method for conferring conformational constraints on peptides. *Biopolymers* **31**, 745–750.
3. Gilon, C., Muller, D., Bitan, G., Salitra, Y., Goldwasser, I. & Hornik, V. (1998) Cycloscan: conformational libraries of backbone cyclic peptides. In *Peptide Chemistry, Structure and Biology* (Ramage, R. & Epton, R., eds), Mayflower Scientific Ltd, XXX, pp. 423–424.
4. Byk, G., Halle, D., Zeltser, I., Bitan, G., Selinger, Z. & Gilon, C. (1996) Synthesis and biological activity of NK-1, selective N-backbone cyclic analogs of the C-terminal hexapeptide of substance P. *J. Med. Chem.* **39**, 3174–3178.

5. Bitan, G., Zeltser, I., Byk, G., Halle, D., Mashriki, Y., Gluhov, E.V., Sukhotinsky, I., Hanani, M., Selinger, Z. & Gilon, C. (1996) Backbone cyclization of the C-terminal part of substance P: the important role of the sulfur in position 11. *J. Peptide Sci.* **2**, 261–269.
6. Bitan, G., Sukhotinsky, I., Mashriki, Y., Hanani, M., Selinger, Z. & Gilon, C. (1997) Synthesis and biological activity of novel backbone-bicyclic substance-P analogs containing lactam and disulfide bridges. *J. Peptide Res.* **49**, 421–426.
7. Gilon, C., Huonges, M., Matha, B., Gellerman, G., Hornik, V., Rosenfeld, R., Afargan, M., Amitay, O., Ziv, O., Feller, E., Gamliel, A., Shohat, D., Wanger, M., Arad, O. & Kessler, H. (1998) A backbone-cyclic receptor 5-selective somatostatin analogue: synthesis, bioactivity and nuclear magnetic resonance conformational analysis. *J. Med. Chem.* **41**, 919–929.
8. Raina, A.K. & Klun, J.A. (1984) Brain factor control of sex pheromone production in the female corn-ear worm moth. *Science* **225**, 531–533.
9. Raina, A.K., Jaffe, H., Kemp, T.G., Keim, P., Blacher, R.W., Fales, H.M., Riley, C.T., Klun, J.A., Ridgway, R.L. & Hayes, T.K. (1989) Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science* **244**, 796–798.
10. Kitamura, A., Nagasawa, H., Kataoka, H., Inoue, T., Matsumoto, S., Ando, T. & Suzuki, A. (1989) Amino acid sequence of pheromone biosynthesis activating neuropeptide (PBAN) of the silkworm *Bombyx mori*. *Biochem. Biophys. Res. Commun.* **163**, 620–526.
11. Kitamura, A., Nagasawa, H., Kataoka, H., Ando, T. & Suzuki, A. (1990) Amino acid sequence of pheromone-biosynthesis-activating neuropeptide-II (PBAN-II) of the silkworm *Bombyx mori*. *Agric. Biol. Chem.* **54**, 2495–2497.
12. Masler, E.P., Raina, A.K., Wagner, R.M. & Kochansky, J.P. (1994) Isolation and identification of a pheromonotropic neuropeptide from the brain–suboesophageal ganglion complex of *Lymantria dispar*: A new member of the PBAN family. *J. Insect Biochem. Molec. Biol.* **24**, 829–836.
13. Duportets, L., Gadenne, C., Dufour, M.C. & Couillaud, F. (1998) The pheromone biosynthesis activating neuropeptide (PBAN) of the black cutworm moth, *Agrotis ipsilon*: immunochemistry, molecular characterization and bioassay of its peptide sequence. *Insect Biochem. Molec. Biol.* **28**, 591–599.
14. Jacquin-Joly, E., Burnet, M., Franxotis, M.C., Ammar, D., Nagnan-Le Meillour, P. & Descoins, C. (1998) cDNA cloning and sequence determination of the pheromone biosynthesis activating neuropeptide of *Mamestra brassicae*: a new member of the PBAN family. *Insect Biochem. Molec. Biol.* **28**, 251–258.
15. Choi, M.Y., Tanaka, M., Kataoka, H., Boo, K.S. & Tatsuki, S. (1998) Isolation and identification of the cDNA encoding the pheromone biosynthesis activating neuropeptide and additional neuropeptides in the oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *Insect Biochem. Molec. Biol.* **28**, 759–766.
16. Davis, M.T.B., Vakharia, V.N., Henry, J., Kemp, T.G. & Raina, A.K. (1992) Molecular cloning of the pheromone biosynthesis-activating neuropeptide in *Helicoverpa zea*. *Proc. Natl Acad. Sci. USA* **89**, 142–146.
17. Kawano, T., Kataoka, H., Nagasawa, H., Isogai, A. & Suzuki, A. (1992) cDNA cloning and sequence determination of the pheromone biosynthesis activating neuropeptide of the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun.* **189**, 221–226.
18. Kawano, T., Kataoka, H., Nagasawa, H., Isogai, A. & Suzuki, A. (1997) Molecular cloning of a new type of cDNA for pheromone biosynthesis activating neuropeptide of the silkworm, *Bombyx mori*. *Biosci. Biotech. Biochem.* **61**, 1745–1747.
19. Ma, P.W.K., Knipple, D.C. & Roelofs, W.L. (1994) Structural organization of the *Helicoverpa zea* gene encoding the precursor protein for pheromone biosynthesis-activating neuropeptide and other neuropeptides. *Proc. Natl Acad. Sci. USA* **91**, 6506–6510.
20. Duportets, L., Gadenne, C., Dufour, M.C. & Couillaud, F. (1999) A cDNA from *Agrotis ipsilon*, that encodes the pheromone biosynthesis activating neuropeptide (PBAN) and other FXPRL peptides. *Peptides* **20**, 899–905.
21. Altstein, M., Gazit, Y. & Dunkelblum, E. (1993) Neuroendocrine control of sex pheromone biosynthesis in *Heliothis peltigera*. *Arch. Insect Biochem. Physiol.* **22**, 153–168.
22. Altstein, M., Dunkelblum, E., Gabay, T., Ben-Aziz, O., Schefler, I. & Gazit, Y. (1995) PBAN induced sex pheromone biosynthesis in *Heliothis peltigera*: structure, dose and time dependent analysis. *Arch. Insect Biochem. Physiol.* **30**, 309–317.
23. Altstein, M., Ben-Aziz, O., Gabay, T., Gazit, Y. & Dunkelblum, E. (1996) Structure function relationship of PBAN/MRCH. In *Insect Pheromone Research: New Directions* (Carde, R.T. & Minks, A.K., eds). Chapman & Hall, XXX, pp. 56–63.
24. Altstein, M., Dunkelblum, E., Gazit, Y., Ben-Aziz, O., Gabay, T., Vogel, Z. & Barg, J. (1997) Structure function analysis of PBAN/MRCH: a basis for antagonist design. In *Modern Agriculture and the Environment* (Rosen, D. et al., eds). Kluwer Academic, XXX, pp. 109–116.
25. Kochansky, J.P., Raina, A.K. & Kemp, T.G. (1997) Structure–activity relationship in C-terminal fragments of pheromone biosynthesis activating neuropeptides in *Helicoverpa zea*. *Arch. Insect. Biochem. Physiol.* **35**, 315–332.
26. Nagasawa, H., Kuniyoshi, H., Arima, R., Kawano, T., Ando, T. & Suzuki, A. (1994) Structure and activity of *Bombyx* PBAN. *Arch. Insect Biochem. Physiol.* **25**, 261–270.
27. Raina, A.K. & Kemp, T.G. (1990) A pentapeptide of the C-terminal sequence of PBAN with pheromonotropic activity. *Insect Biochem.* **20**, 849–851.
28. Raina, A.K. & Kemp, T.G. (1992) Structure activity studies of PBAN of *Helicoverpa zea* (Lepidoptera: Noctuidae). *Insect Biochem. Molec. Biol.* **22**, 221–225.
29. Nachman, R.J., Holman, M.G. & Haddon, W.F. (1993) Leads for insect neuropeptide mimetic development. *Arch. Insect Biochem. Physiol.* **22**, 181–197.
30. Schoofs, L., De Vanden, J.B. & Loof, A. (1993) The myotropic peptides of *Locusta migratoria*: structure, distribution, function and receptors. *Insect Biochem. Molec. Biol.* **23**, 859–881.
31. Matsumoto, S., Fonagy, A., Kurihara, M., Uchiumi, K., Nagamine, T., Chijimatsu, M. & Mitsui, T. (1992) Isolation and primary structure of a novel pheromonotropic neuropeptide structurally related to leucopyrokinin from the armyworm larvae, *Pseudaletia separata*. *Biochem. Biophys. Res. Commun.* **182**, 534–539.
32. Imai, K., Konno, T., Nakazawa, Y., Komiya, T., Isobe, M., Koga, K., Goto, T., Yaginuma, T., Sakakibara, K., Hasegawa, K. & Yamashita, O. (1991) Isolation and structure of diapause hormone of the silkworm, *Bomyx mori*. *Proc. Japan Acad. Series B* **67**, 98–101.
33. Altstein, M., Gazit, Y., Ben-Aziz, O., Gabay, T., Marcus, R., Vogel, Z. & Barg, J. (1996) Induction of cuticular melanization in *Spodoptera littoralis* larvae by PBAN/MRCH: development of a quantitative bioassay and structure function analysis. *Arch. Insect Biochem. Physiol.* **31**, 355–370.

34. Matsumoto, S., Kitamura, A., Nagasawa, H., Kataoka, H., Orikasa, C., Mitsui, T. & Suzuki, A. (1990) Functional diversity of a neurohormone produced by the suboesophageal ganglion: molecular identity of melanization and reddish coloration hormone and pheromone biosynthesis activating neuropeptide. *J. Insect Physiol.* **36**, 427–432.
35. Schoofs, L., Holman, M.G., Nachman, R.J., Hayes, T.K. & DeLoof, A. (1991) Isolation, primary structure and synthesis of locustapyrokinin: a myotropic peptide of *Locusta migratoria*. *Gen. Comp. Endocrinol.* **81**, 97–104. 4
36. Nachman, R.J., Holman, M.G. & Cook, B.J. (1986) Active fragments and analogs of the insect neuropeptide leucopyrokinin: structure–function studies. *Biochem. Biophys. Res. Commun.* **137**, 936–942.
37. Nachman, R.J., Zdareej, J., Holman, M.G. & Hayes, T.K. (1997) Pupariation acceleration in fleshfly (*Sarcophaga bullata*) larvae by the pyrokinin/PBAN neuropeptide family. *Ann. N.Y. Acad. Sci.* **814**, 73–79.
38. Gazit, Y., Dunkelblum, E., Benichis, M. & Altstein, M. (1990) Effect of synthetic PBAN and derived peptides on sex pheromone biosynthesis in *Heliothis peltigera* (Lepidoptera: Noctuidae). *Insect Biochem.* **20**, 853–858.
39. Altstein, M., Ben-Aziz, O., Daniel, S., Schefler, I., Zeltser, I. & Gilon, C. (1999) Backbone cyclic peptide antagonists, derived from the insect pheromone biosynthesis activating neuropeptide, inhibit sex pheromone biosynthesis in moths. *J. Biol. Chem.* **274**, 17573–17579. 5
40. Gilon, C., Zeltser, I., Daniel, S., Ben-Aziz, O., Schefler, I. & Altstein, M. (1997) Rationally designed neuropeptide antagonists: a novel approach for generation of environmentally friendly insecticides. *Invert. Neurosci.* **3**, 245–250.
41. Zeltser, I., Gilon, C., Ben-Aziz, O., Schefler, I. & Altstein, M. (2001) Discovery of a linear antagonist to the insect pheromone biosynthesis activating neuropeptide (PBAN). *Peptides* in press.
42. Muller, D., Zeltser, I., Bitan, G. & Gilon, C. (1997) Building units for N-backbone cyclic peptides. 3. Synthesis of protected N^{α}-(ω-aminoalkyl) amino acids and N^{α}-(ω-carboxyalkyl) amino acids. *J. Org. Chem.* **62**, 411–416.
43. Houghten, R. (1985) General method for the rapid solid phase synthesis of large numbers of peptides: specificity of antigen–antibody interaction at the level of individual amino acids. *Proc. Natl Acad. Sci. USA* **82**, 5131–5135.
44. Bennick, A. (1987) Structural and genetic aspects of proline rich protein. *J. Dent. Res.* **66**, 457–461.
45. Shivanandaiah, K.M., Sureshbabu, V.V. & Shankaramma, S.C. (1995) Solid-phase synthesis of oxytocin using iodotrichlorosilan as Boc deprotecting agent. *Int. J. Peptide Protein Res.* **45**, 377–379.
46. Efron, L. & Gilon, C. (1998) The use of iodotrichlorosilane for the synthesis of cyclic peptides. In *Peptides 1996, Proceedings of the Twenty-Fourth European Peptide Symposium* (Ramage, R. & Epton, R., eds). Xxxxxxxxxxx, Xxxxxxxxxxx, pp. 357–358.
47. Hruby, V.J., Al-Obeidi, F. & Kazmierski, W. (1990) Emerging approaches in the molecular design of receptor-selective peptide ligands: conformation, topological and dynamic considerations. *Biochem. J.* **268**, 249–262.
48. Hruby, V.J. (1992) Strategies in the development of peptide antagonists. *Prog. Brain Res.* **92**, 215–224.
49. Nachman, R.J., Roberts, V.A., Dyson, H.J., Holman, M.G. & Tainer, J.A. (1991) Active conformation of an insect neuropeptide family. *Proc. Natl Acad. Sci. USA* **88**, 4518–4522.
50. Golic-Gradadolnik, S., Mierke, D.F., Byk, G., Zeltser, I., Gilon, C. & Kessler, H. (1994) Comparison of the conformations of active and nonactive backbone cyclic analogs of substance P as a tool to elucidate features of the bioactive conformation: NMR and molecular dynamics in water and dimethylsulfoxide. *J. Med. Chem.* **37**, 2145–2152.
51. Saulitis, J., Mierke, D.F., Byk, G., Gilon, C. & Kessler, H. (1992) Conformation of cyclic analogues of substance P: NMR and molecular dynamics in demethylsulfoxide. *J. Am. Chem. Soc.* **114**, 4818–4823.
52. Kasher, R., Oren, D.S., Barda, Y. & Gilon, C. (1999) Protein miniaturization: the backbone cyclic proteinomimetic approach. *J. Mol. Biol.* **292**, 421–429.

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