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Backbone Cyclic Peptide Antagonists, Derived from the Insect Pheromone Biosynthesis Activating Neuropeptide, Inhibit Sex Pheromone Biosynthesis in Moths*

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We describe an application of the backbone cyclization and cycloscan concept for the design and synthesis of pheromone biosynthesis activating neuropeptide (PBAN) antagonists capable of inhibiting sex pheromone biosynthesis in *Heliothis peltigera* female moths. Two backbone cyclic (BBC) sub-libraries were designed and synthesized. The structure of the first sub-library ([Arg^{27}]PBAN27–33NH$_2$, termed the Ser sub-library) was based on the active C-terminal hexapeptide sequence (Tyr-Phe-Ser-Pro-Arg-Leu-NH$_2$) of PBAN1–33NH$_2$, which was found to comprise its active core. The second sub-library ([Arg^{27},D-Phe30]PBAN27–33NH$_2$, termed the D-Phe sub-library) was based on the sequence of the lead antagonist Arg-Tyr-Phe-(D)Phe-Pro-Arg-Leu-NH$_2$. In both sub-libraries the Pro residue was replaced by an N^{α}(ω-amino-alkyl)Gly building unit having various lengths of the alkyl chain. All the cyclic peptides in each sub-library had the same primary sequence and the same location of the ring. The members of each library differed from each other by the bridge size and bridge chemistry. Screening of the two libraries for pheromonotropic antagonists resulted in the disclosure of four compounds that fully inhibited sex pheromone biosynthesis at 1 nmol and were devoid of agonistic activity. All antagonistic peptides originated from the D-Phe sub-library. Substitution of the D-Phe30 amino acid with a Ser resulted in a loss of antagonistic activity. Agonistic activities were exhibited by peptides from both sub-libraries.

It is well established that the activation of multiple receptors by the same peptide arises from the ability of a given peptide to exist in different interchangeable bioactive conformations (1). To attain receptor selectivity it is, therefore, essential to restrict the conformational space of a peptide so it can attain one bioactive conformation.

Cyclization is an important and attractive way to restrict the conformational space of peptidic structures (1). Conformationally restricted peptides containing medium and long range cyclizations have been mainly prepared following the same modes of cyclization of naturally occurring peptides. These include end-to-end, side-chain-to-side-chain, and side-chain-to-end (2). These modes of cyclization involve modifications of side chains and ends which in many cases are essential for bioactivity, and therefore their modification causes loss of activity. Also, these modes of cyclization are not sufficient to screen effectively the conformational space available for a linear peptide with a given sequence. To overcome these limitations, we have introduced two methods called backbone cyclization (2) and cycloscan (3).

Backbone cyclization is a general method by which a conformational constraint is imposed on peptides through the connection of the N^{α} and/or C^{α} atoms in the peptide backbone to each other or to side chains or to the carboxyl and amino ends (2). Thus, the cyclization can be performed while retaining the functionality and the activity of the side chains. Backbone cyclization allows nine new modes of cyclization in addition to the four modes in naturally occurring peptides. Preparation of backbone cyclic (BBC)[1] peptides involves the use of a large variety of orthogonally protected N^{α} and C^{α} (ω-amino-, ω-carboxy-, and ω-thio-alkyl) amino acids building units (4–6). Synthetic procedures have been developed to incorporate these building units into peptides using the solid phase methodology (7, 8).

The advantages of backbone cyclization over the naturally occurring modes of cyclization are because of the immense variability of spatial orientation of the constitute residues which result from the multiple anchoring points within a chain or between chains. This allows us to screen the conformational space of a given peptide in an extremely efficient manner. Furthermore, most of the modes of backbone cyclization do not involve chemical modifications of side chains which are essential for biological activity. Backbone cyclization has been shown previously to convert peptides into selective and metabolically stable peptidomimetics with enhanced biological activity as compared with the linear parent peptide (7–10).

A rapid method for the disclosure of a BBC peptide that closely resembles a unique agonistic or antagonistic bioactive conformation can be obtained by the screening of BBC libraries (cycloscan) (3). Two such libraries were described: biased and random. In our study we used the biased library approach. The biased library is composed of a BBC peptide in which the primary sequence of the active linear peptide is retained and the members of the library differ solely in their conformation. The diversity of the biased library (also termed "sequence-biased cycloscan") resides in: (i) the modes of backbone cyclization (2); (ii) the position of the backbone bridge along the peptide sequence; (iii) the size of the bridge; and (iv) the chemistry of the bridge. Biased libraries of BBC peptide allow sys-

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[1] The abbreviations used are: BBC, backbone cyclic; PBAN, pheromone biosynthesis activating neuropeptide; SAR, structure-activity relationship; PK, pyrokinin; TFA, trifluoroacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography.

tematic screening of broad ranges of the conformational space available to a given linear peptide.

In this study we present the application of sequence-biased cycloscan for the disclosure of small BBC agonists and antagonists of the insect pheromone biosynthesis activating neuropeptide (PBAN) which regulates, among other functions (for a review, see Ref. 11), sex pheromone biosynthesis in female moths (12–14).

Sexual communication between sexes in Lepidopteran species is mediated mainly by sex pheromones (15, 16). Sex pheromones are volatile compounds that are used by Lepidopteran insects to attract potential mates from a distance. In Lepidoptera, sex pheromones are synthesized and secreted from specialized glandular cells that are located in the inter-segmental membrane between the eighth and ninth abdominal segments (17). Sex pheromones play an important role in the elicitation of mating behavior in moths and are, therefore, crucial for successful mating and maintenance of reproductive isolation. Understanding the mechanisms that underlie sex pheromone production is of major interest and importance.

In 1984 it was first reported (12) that sex pheromone production in *Helicoverpa zea* female moths is controlled by a cerebral neuroendocrine factor which was termed PBAN. Since then, the presence of PBAN has been demonstrated in a variety of moths as well as in non-lepidopteran species, and its mode of action has been studied in many laboratories (for review, see Refs. 11, 13, and 14). To date, four different PBAN molecules have been isolated from three different moth species, and their primary structures have been determined (18–21). Examination of the primary structure of all four peptides revealed that they share a high degree of homology.

Detailed structure-activity relationship studies (SAR), using synthetic PBAN and shorter peptides derived from its sequence, have revealed that the C-terminal pentapeptide, Phe-Xxx-Pro-Arg-Leu-NH_2 (Xxx = Ser), which is identical in all PBAN molecules, is the active core required for biological activity (22–31). In addition, it has been found that this C-terminal pentapeptide (where Xxx = Ser, Gly, Thr, Val) is homologous with the C-terminal pentapeptide sequence of other families of insect neuropeptides: the pyrokinins (PK) and the myotropins (MT) (myotropic peptides isolated from *Leucophaea maderae* and *Locusta migratoria*) (32–39), and also with the C-terminal region of *Pseudaletia separata* pheromonotropin (Pss-PT) (40) and *Bombyx mori* diapause hormone (Bom-DH) (41).

The above mentioned group of neuropeptides has been designated recently the pyrokinin/PBAN family. The members of this family have been found to be responsible for a variety of physiological and behavioral functions such as: contraction of the locust oviduct (32), myotropic activity of the cockroach and locust hindgut (32, 42), egg diapause in the silkworm (41), and acceleration of pupariation in the fleshfly larvae (43), in addition to stimulation of sex pheromone biosynthesis in female moths (12–14, 44) and melanization and reddish coloration in moth larvae (23, 45).

Despite the intensive studies of the bio-activity of this family of peptides with respect to sex pheromone biosynthesis and the other functions, very little is known about the endogenous mechanism and the structural, chemical, and cellular basis of their activity. It is still not known which endogenous peptide(s) mediates each of these functions *in vivo*, whether each function is mediated by a different peptide and if each peptide mediates one or several functions. It is also not clear which receptors mediate these functions, what are their characteristics, and whether the receptors share functional homologies. A promising approach for resolving these issues is the use of receptor-selective agonists and/or antagonists, as was demonstrated previously for vertebrate neuropeptides (46–49). Backbone cyclization and cycloscan provides a good solution for this issue by introducing conformational constraint into peptides and thus, acquiring them with higher selectivity because of the restricted conformational space which can be recognized only by one receptor. Backbone cyclization also acquires peptides with higher potency, enhanced metabolic stability, and improved bioavailability. The advantages introduced by backbone cyclization and cycloscan as well as the availability of bioassays for the pyrokinin/PBAN family of peptides (23, 39, 43, 44) (which are required to obtain the essential information for the design of BBC peptides and specificity for the desired biological effects), led us to combine both approaches for the generation of receptor-selective agonists and antagonists.

In this study we report the generation of highly potent, conformationally constrained BBC heptapeptide agonists and antagonists which fully inhibit one of the functions mediated by PBAN: sex pheromone biosynthesis in the female moth *Heliothis peltigera*.

MATERIALS AND METHODS

General—Fmoc-protected amino acids with standard side-chain protecting groups as well as resins and reagents for peptide synthesis were obtained from Novabiochem (Laufelingen, Switzerland). Ultrapure quality solvents were obtained from Basker. Other reagents were obtained from Aldrich. Building units for BBC peptides were prepared as described previously (6).

Synthesis of BBC Peptides—BBC peptide libraries were synthesized as described before (9). Briefly, peptides were synthesized by the Simultaneous Multiple Peptide Synthesis methodology (50) on Rink Amide 4-methylbenzhydrylamine Resin (loading 0.55 mmol/g) using Fmoc chemistry and *N*-methylpyrrolidone as solvent. The following BBC building units were used: *t*-butoxycarbonyl (Boc)-NH$(CH_2)_n$-*N*(Fmoc) CH_2-COOH (n = 2, 3, 4, 6). Couplings were performed for 2 h by a preactivated solution of 3-fold excess of Fmoc amino acids or building units with bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBrOP) and 6-fold excess of diisopropylethylamine for 5 min in *N*-methylpyrrolidone. Deprotection of the Boc protecting group from the *N*(amino alkyl) group of the building units was carried out with Cl_3SiI, prepared *in situ* from $SiCl_4$ and NaI in ACN: dichloromethane (1:1). On resin, cyclization was achieved by 6-fold excess of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and repeated four times. The peptides were removed from the resin with concomitant side chain deprotection by 90% trifluoroacetic acid (TFA) with addition of scavengers (H_2O:ethanedithiol:thioanisole, 2.5:2.5:5%). The resin was removed by filtration and the TFA evaporated to dryness by stream of nitrogen. The residue was extracted three times with ether to remove the scavengers. The dry crude peptide was dissolved in water and lyophilized. The crude peptides were purified by preparative HPLC and characterized by mass spectrometry and amino acid analysis of hydrolisates. All peptides gave the expected mass and were pure by analytical HPLC. Analytical data are shown in Tables I and II.

Synthesis of Hez-PBAN—Hez-PBAN (PBAN1–33NH_2) (18) was synthesized by the Merrifield Solid-Phase Peptide Synthesis method (51), on an ABI peptide synthesizer Model 433A, on Rink Amide 4-methylbenzhydrylamine Resin (loading 0.55 mmol/g). The peptide was synthesized by the FastMoc™ methodology and removed from the resin with concomitant side chain deprotection by 90% TFA with addition of scavengers (H_2O:ethanedithiol:thioanisole, 2.5:2.5:5%). Peptide purity was assessed by reverse phase-HPLC (see legend to Table I) and found to be 95%.

Pheromonotropic Bioassay—H. peltigera moths were reared on an artificial diet as described previously (52).

Determination of Agonistic and Antagonistic Activity—Agonistic and antagonistic activities of the BBC peptides were determined as described previously (44). Agonistic activity of BBC peptides was determined by monitoring the ability of the injected peptides (at 1 nmol, unless otherwise indicated) to induce sex pheromone biosynthesis in females (in the absence of PBAN1–33NH_2). Females injected with 1 nmol (unless otherwise indicated) of PBAN1–33NH_2 served as a reference for agonistic activity. Antagonistic activity was determined by monitoring the ability of the BBC peptides (at 1 nmol) to inhibit sex pheromone biosynthesis evoked by 0.5 pmol of exogenously injected

TABLE I
Analytical data of the Ser sub-library BBC peptides

Peptide No. (n + m)[a]	MS[b] Found	MS Calculated	HPLC[c] *t*	Purity	Amino Acid analysis				
					Leu	Arg	Phe	Ser	Tyr
			min	%					
4 (2 + 2)	1122.6	1122.2	25.75	83	1	1.81	0.82	0.86	0.9
5 (2 + 3)	1037.1	1036.2	24.96	85	1	1.67	0.94	0.96	0.84
6 (2 + 4)	1051.2	1050.3	26.06	73	1	1.83	0.99	1.07	0.85
7 (3 + 2)	1037.2	1036.2	24.75	97	1	1.98	0.93	0.87	0.97
8 (3 + 3)	1051.3	1050.3	24.84	90	1	1.55	0.85	1.03	0.83
9 (3 + 4)	1064.8	1064.3	25.47	73	1	1.62	0.93	0.94	0.91
10 (4 + 2)	1050.9	1050.3	24.56	94	1	2.09	0.99	0.91	1.19
11 (4 + 3)	1065.1	1064.3	24.86	84	1	2.19	1.14	1.14	1.21
12 (4 + 4)	1079.3	1078.3	26.15	79	1	1.90	1.04	1.10	1.12
13 (6 + 2)	1079.0	1078.3	26.50	97	1	2.08	0.98	0.95	1.03
14 (6 + 3)	1099.6	1092.3	26.03	85	1	1.79	0.99	1.06	0.91
15 (6 + 4)	1107.0	1106.4	26.62	73	1	1.76	0.70	1.10	0.80

[a] See Fig. 1*A*.
[b] MS, mass spectrometry.
[c] Preparative and semipreparative purification of crude peptides was performed by reverse-phase high pressure liquid chromatography (RP-HPLC) on C8 columns obtained from Merck (Darmstadt, Germany) (semi-preparative flow 4.5 ml/min and preparative flow 9 ml/min) using the following gradient of *A* = acetonitrile (ACN) and *B* = H_2O + 0.1% trifluoroacetic acid: 0–5 min 15% A + 85% B; 30 min 50% A + 50% B; 40–50 min 100% A. The purity of the peptides was assessed on C18 analytical RP-HPLC columns obtained from Vidac using the following gradient of *A* = ACN and *B* = H_2O (+0.1% trifluoroacetic acid): 0–5 min 5% A + 95% B; 30 min 50% A + 50% B; 40–50 min 100% A. The peptides were characterized by time of flight mass spectrometry (TOF-MS) and electrospray mass spectrometry (ES-MS) and by amino acid analysis of hydrolysates.

PBAN1–33NH_2. Females injected with 0.5 pmol of PBAN1–33NH_2 served as a reference for maximal stimulation, and those injected with 0.1 M phosphate buffer served to determine the basal pheromone biosynthesis at photophase (which did not exceed 20 ng/female). Pheromone glands were excised 2 h post-injection, and sex pheromone was extracted and quantified by capillary gas chromatography as described previously (22). All experiments were performed with a minimum of ten females per treatment.

RESULTS

Design of BBC PBAN-derived Sub-libraries—Detailed SAR studies, using synthetic Hez-PBAN and shorter peptides derived from its sequence, revealed that the C-terminal pentapeptide/PBAN that is common to all members of the pyrokinin/PBAN family comprises the active core required for biological activity (22–31). Furthermore, studies performed in our laboratory indicated that, under certain conditions, the hexapeptide sequence derived from Hez-PBAN (PBAN28–33NH_2: Tyr-Phe-Ser-Pro-Arg-Leu-NH_2, MINI-PBAN) is as active as the full-length PBAN (22). Based on this sequence, a biased library of linear peptides has been designed, and a linear lead antagonist ([Arg^{27}-D-Phe^{30}]PBAN27–33NH_2: Arg-Tyr-Phe-(D)Phe-Pro-Arg-Leu-NH_2) has been disclosed (53). These findings set the basis for the design and synthesis of two sub-libraries of cyclic peptides. The structure of the first sub-library (termed the Ser sub-library, see Fig. 1*A*) was based on a slight modification of MINI-PBAN ([Arg^{27}]PBAN27–33NH_2). The second sub-library, [Arg^{27}-D-Phe^{30}]PBAN27–33NH_2 (termed the D-Phe sub-library, see Fig. 1*B*), was based on the sequence of the lead antagonist. In both sub-libraries, following the hierarchical approach for the discovery of BBC leads, the Pro residue was replaced by the N^{α}(ω-amino-alkyl)Gly building unit having various lengths of the alkyl chain (Fig. 1, *n* = 2–4, 6). The ω-amino group of the Gly building unit was connected to the N-terminal amino group by a dicarboxylic acid spacer (Fig. 1, *m* = 2–4). All the cyclic peptides in each sub-library had the same primary sequence and the same location of the ring. The members of each library differed from each other by the bridge size and the position of the amide bond along the bridge. The two sub-libraries were tested for their agonistic and antagonistic activity using a pheromonotropic bioassay that was optimized by us for *H. peltigera* (44). The aim of the experiments was to discover potent pheromonotropic BBC antagonist(s) devoid of agonistic activity and to determine the structural requirements for the agonistic and antagonistic activities. These SAR studies were also performed to assist in the further design of improved non-peptidic PBAN agonists and antagonists.

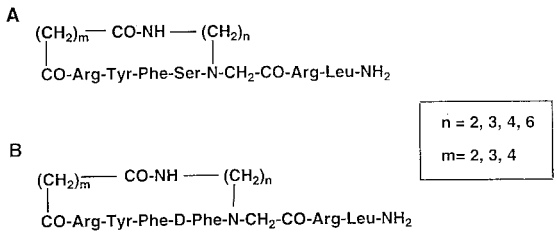

FIG. 1. **General structure of the BBC Ser (*A*) and the D-Phe (*B*) sub-libraries of peptides**.

Analysis of the Antagonistic and Agonistic Activities of the BBC Peptides—The first part of the study involved examination of the ability of BBC peptides from the D-Phe sub-library (numbered 19–30, Table II) to inhibit sex pheromone biosynthesis evoked by exogenously administered (injected) PBAN1–33NH_2 to adult female moths. The data in Fig. 2 depict the presence of five potent antagonistic peptides (numbers 20, 21, 22, 25, and 28) exhibiting over 50% inhibitory activity (at 1 nmol). The inhibitory potency of these peptides ranged from 55% (peptide 25) to a maximum of 96% (peptide 20).

Antagonists may be devoid of agonistic activity, or exhibit full or partial agonistic activity. Backbone cyclization can, in principle, convert an antagonistic linear peptide into an agonist and *vice versa*. Therefore, the antagonistic peptides (numbers 20, 21, 22, 25, and 28) were tested for their agonistic activity at the same concentration used to assess their antagonistic activity (at 1 nmol). Agonistic activity was determined by the ability of the peptides to evoke sex pheromone biosynthesis in *H. peltigera* in the absence of PBAN1–33NH_2. Three of the five antagonists (numbers 22, 25, and 28) were devoid of agonistic activity (Fig. 3); peptide 20 had minor agonistic activity (10%); only one peptide (number 21) exhibited high agonistic activity (62%) at 1 nmol.

Synthetic PBAN1–33NH_2 activates pheromone biosynthesis at doses as low as 0.3 pmol (44). Many neuropeptides are known to cause desensitization of their receptors at concentrations above their EC_{50}. To exclude the possibility that the lack of agonistic activity (at 1 nmol) of the above mentioned antag-

TABLE II
Analytical data of the D-Phe sub-library BBC peptides

Peptide No. $(n + m)^a$	MS[b] Found	MS Calculated	HPLC[c] *t*	Purity	Amino Acid analysis			
					Leu	Arg	Phe	Tyr
			min	%				
19 (2 + 2)	1082.8	1082.3	29.51	90	1	1.77	1.83	0.88
20 (2 + 3)	1098.1	1096.3	29.78	97	1	1.76	1.98	0.81
21 (2 + 4)	1110.8	1110.4	29.60	92	1	1.72	1.92	0.83
22 (3 + 2)	1099.5	1096.3	27.80	98	1	1.93	1.88	0.85
23 (3 + 3)	1112.7	1111.4	29.54	96	1	1.88	1.94	0.95
24 (3 + 4)	1125.0	1124.4	29.80	79	1	1.84	1.93	0.92
25 (4 + 2)	1110.7	1110.4	30.11	75	1	2.38	2.37	1.00
26 (4 + 3)	1125.8	1124.4	30.68	80	1	1.57	1.62	0.77
27 (4 + 4)	1139.4	1138.4	30.24	88	1	1.73	1.84	0.87
28 (6 + 2)	1138.9	1138.4	31.64	92	1	1.74	1.90	0.84
29 (6 + 3)	1153.1	1152.4	30.79	79	1	2.09	2.02	0.96
30 (6 + 4)	1167.6	1166.4	31.58	80	1	1.91	1.97	0.94

[a] See Fig. 1*B*.
[b] MS, mass spectrometry.
[c] For details, see Table I.

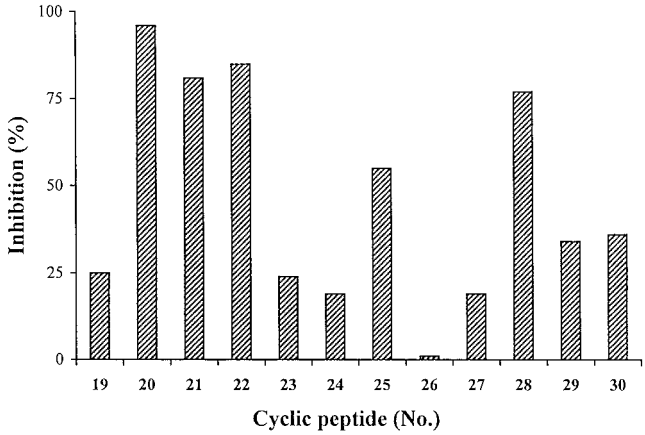

FIG. 2. **Inhibition of sex pheromone biosynthesis in *H. peltigera* females by cyclic peptides from the D-Phe sub-library.** Peptides were injected to *H. peltigera* females at a dose of 1 nmol together with 0.5 pmol of PBAN1–33NH_2. Glands were excised 2-h post-injection, and pheromone content was determined by capillary gas chromatography as described under "Materials and Methods." The degree of inhibition of each peptide is expressed as 100 minus the ratio (in percentage) between the pheromone content in the gland evoked by the injection of PBAN1–33NH_2 in the presence and absence of each of the peptides. The amount of sex pheromone evoked by 0.5 pmol of PBAN1–33NH_2 ranged from 93 to 113 ng of pheromone/female. Pheromone content was monitored with at least ten females for each of the tested peptides.

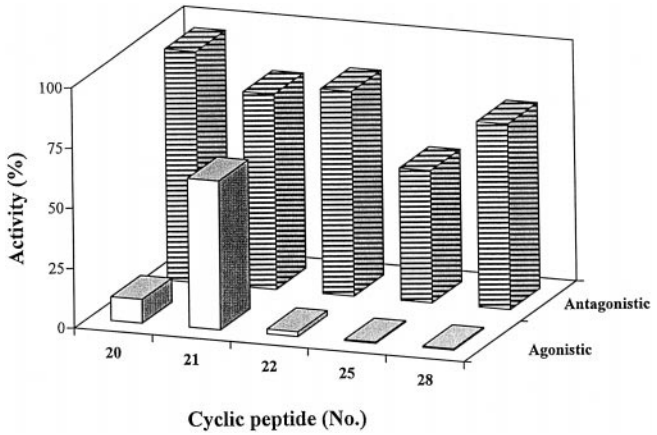

FIG. 3. **Comparison of agonistic and antagonistic activities of cyclic peptides from the D-Phe sub-library.** Peptides were injected at a dose of 1 nmol. Agonistic activity was determined in *H. peltigera* females by the injection of each of the peptides for 2 h; antagonistic activity was determined as described in the legend to Fig. 2. Agonistic activity is expressed as the ratio (in percentage) between the sex pheromone content evoked by the injection of each of the cyclic peptides and PBAN1–33NH_2 (at 1 nmol). The amount of sex pheromone evoked by 1 nmol of PBAN1–33NH_2 ranged from 93 to 198 ng pheromone/female.

onists is not because of desensitization, their agonistic activity was measured at lower concentrations (*e.g.* 1, 10, and 100 pmol). All tested peptides (numbers 20, 22, 25, and 28) exhibited very low agonistic activity at all tested concentrations (less than 10% of the activity evoked by PBAN1–33NH_2, at the same concentration). All other peptides from the D-Phe sub-library (numbers 19, 23, 24, 26, 27, 29, and 30, Table II) that exhibited low or no antagonistic activity also failed to stimulate sex pheromone biosynthesis in *H. peltigera* females (at 1 nmol). The amount of pheromone that was found in females injected with these peptides was negligible and ranged from 0 to a maximum of 3 ng/gland.

Further studies involved examination of the ability of the peptides from the Ser sub-library (numbered 4–15, Table I) to evoke agonistic activity. The data in Fig. 4 depict one relatively potent agonist (number 14) which exhibits 75% activity, at 1 nmol, as compared with the activity of 1 nmol PBAN1–33NH_2, and the presence of two other peptides (numbers 6 and 10) with low agonistic activity (33 and 45%, as compared with the activity of 1 nmol PBAN1–33NH_2, respectively). All other peptides in the Ser sub-library had low or no agonistic activity compared with that of PBAN1–33NH_2, when injected for 2 h. Examination of the activity of peptides 6, 10, and 14 at lower doses (1, 10, and 100 pmol) revealed very low activity at all tested concentrations. The only peptide that exhibited any activity was number 14 at 100 pmol. The activity was similar to that exhibited by the linear peptides PBAN28–33NH_2 and [Arg^{27}]PBAN27–33NH_2 and lower than that of PBAN1–33NH_2 (data not shown).

Based on the same rationale as was mentioned above on the ability of peptides to exhibit both agonistic and antagonistic activities, peptides of the Ser sub-library (that did not exhibit agonistic activity) were tested for their ability to act as antagonists, namely, to inhibit sex pheromone biosynthesis evoked by exogenously administered PBAN1–33NH_2. Pheromone biosynthesis was evoked by 0.5 pmol of PBAN1–33NH_2, and each peptide from the Ser sub-library (numbers 4, 5, 7–9, and 11–13, Table I) was tested for its antagonistic activity at a concentration of 1 nmol. None of the tested peptides exhibited any antagonistic activity under these experimental conditions.

The detailed SAR studies that were performed with the BBC peptides from both libraries lead to the conclusion that peptides from the Ser sub-library were devoid of any pheromono-

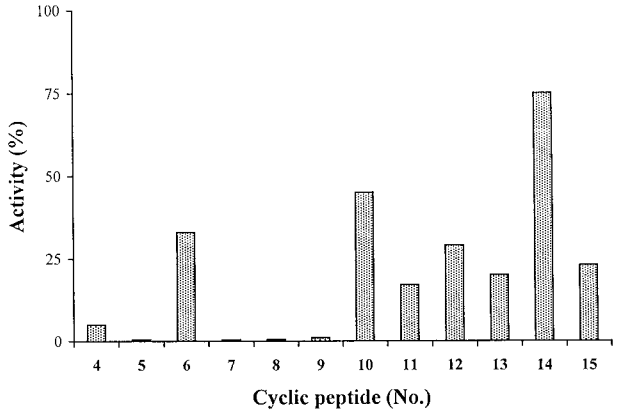

FIG. 4. **Agonistic activity of cyclic peptides from the Ser sub-library.** All experimental details are as described in the legend to Fig. 3. Agonistic activity is expressed as the ratio (in percentage) between the sex pheromone content evoked by the injection of each of the cyclic peptides and PBAN1–33NH_2 (at 1 nmol for 2 h). The amount of sex pheromone evoked by 1 nmol of PBAN1–33NH_2 ranged from 86 to 191 ng of pheromone/female.

tropic antagonistic activity and that antagonistic peptides were obtained only from the D-Phe sub-library (peptides 20, 21, 22, 25, and 28) (Table III). In addition, it was found that agonistic or partial agonistic activities were exhibited by peptides from both sub-libraries (numbers 6, 10, and 14 from the Ser sub-library and 21 from the D-Phe sub-library). Examination of the correlation between the bridge size and activity revealed that six of the eight active peptides (whether agonistic or antagonistic) had a bridge size of $m + n = 5$ or 6, and peptides with a bridge size of $m + n = 4$, 7, and 10 were inactive. However, not all peptides with a bridge size of $m + n = 6$ exhibited activity (*e.g.* peptides 8 and 23, Table III), and in general only 8 of the 24 BBC peptides described herein were found to be agonists and/or antagonists. This is despite the fact that all of the peptides within each library had the same parent primary sequence (either the agonistic MINI-PBAN sequence—Ser sub-library, or the linear lead antagonistic sequence—D-Phe sub-library). Moreover, in some cases the biological activity was determined by the position of the amide bond along the bridge. Peptides 21 and 25 ($n = 2$; $m = 4$ and $n = 4$; $m = 2$, respectively) were active, whereas peptide 23, which had the same sequence, the same location of the ring in the sequence, and the same ring size, but differed only in the position of the ring amide bond ($n = 3$; m = 3), was completely inactive. Similar results were obtained with peptides 27 and 28 (a combination of $n = 4$ and $m = 4$ was inactive, whereas $n = 6$ and $m = 2$ was active; Table III). We assume that the exact position of the bridge amide bond is critical for the stabilization of the bioactive conformation by intramolecular hydrogen bonding. The differences in agonistic and/or antagonistic potencies exhibited by the various BBC peptides within each sub-library illustrate the crucial importance of conformation because all the members of each sub-library have the same parent sequence.

DISCUSSION

Selective peptide receptor antagonists are most valuable tools for understanding the detailed pharmacology of peptide-receptor systems. Selective antagonists of insect neuropeptides having the appropriate properties of selectivity, metabolic stability, and bioavailability are also good candidates for potential use as insecticides. Despite their tremendous importance, no methodology is available for a *de novo* design of selective antagonists to a certain receptor. Moreover, there is no way to predict *a priori* which "new" ligand-receptor interactions will lead to antagonists or to agonists of greater or lesser potency. The basic problem for the design of antagonists is to obtain a lead compound. Once an antagonistic lead compound is obtained, its activity can be improved by conformational constraints in combination with SAR studies.

Potent peptide antagonists have been obtained, so far, by two main strategies: (*a*) antagonists derived from agonists, where the lead antagonists are obtained by serendipity during systematic SAR studies of the endogenous agonist; and (*b*) non-peptide antagonists, where lead compounds were obtained "incidentally" or by trial and error from natural product or chemical libraries by multi-receptor screening (54). We have chosen to apply the first approach. In a previous study we elucidated the active sequence of PBAN by systematic reduction of the peptide sequence by one or several amino acid residues at a time from the N and the C termini to determine the minimum active sequence necessary for bioactivity. This study resulted in the disclosure of a sequence of six amino acids derived from the C terminus of the peptide (Tyr-Phe-Ser-Pro-Arg-Leu-NH_2) that was equipotent with PBAN1–33NH_2 (22). Then, we systematically replaced L-amino acids by hydrophobic D-amino acids (*e.g.* D-Phe) in the MINI-PBAN sequence, which led to the disclosure of a linear lead antagonist (53). The same approach led in the past to the disclosure of antagonists of several neuropeptides such as: bombesin (55), substance P (56), neurokinin A (57), and vasoactive intestinal peptide (58). For review, see Refs. 59 and 60.

To obtain improved antagonists that exhibit selectivity, metabolic stability, and bioavailability, we applied backbone cyclization and sequence-biased cycloscan using the amino acid sequence of the linear antagonist as a lead sequence. Two BBC sub-libraries were generated, out of which one agonist and four potent anti-PBAN antagonists (devoid of agonistic activity) capable of inhibiting sex pheromone biosynthesis in *H. peltigera* females were disclosed. The conformational constraint that was imposed on the BBC peptides in both sub-libraries enabled only a few of the BBC peptides to adapt to the appropriate bioactive conformation, even though all the peptides were based on highly active primary sequences, further strengthening the importance of conformational compatibility.

The BBC approach, which introduces conformational constraint on peptides and acquires metabolic stability for them, introduces many advantages in the design of agonists and antagonists. First, it provides a tool to disclose selective agonists and antagonists, as was proven in our laboratory in the case of substance P (7) and somatostatin (8), where the application of backbone cyclization resulted in highly selective analogs for the neurokinin receptors (NK-1) and somatostatin receptors SSTR2 and SSTR5, respectively. This approach also facilitates determination of the bioactive conformation of PBAN and the conformational requirements for pheromonotropic agonistic and antagonistic activities that can be assessed by nuclear magnetic resonance (NMR) studies (8, 61). This information is essential for the further design of improved peptidomimetic and non-peptide agonists and antagonists of PBAN with higher potency, stability, and improved bioavailability. The metabolic stability that is acquired for the BBC peptides is also of major importance especially for *in vivo* studies, as has been proven in our laboratory using the above anti-PBAN antagonists. Preliminary studies indicate that the BBC peptides exhibit protracted metabolic stability ($t_{1/2} = 160$ min compared with $t_{1/2} = 7$ min of the linear analog [Arg^{27}]PBAN28–33NH_2), and their injection to *H. peltigera* females at scotophase resulted in a marked decrease in pheromone biosynthesis (that lasted over 10 h) evoked by endoge-

TABLE III
Summary of the biological activities of the D-Phe and Ser BBC peptides

Bridge size[a]			D-Phe sub library			Ser sub-library		
n	m	m + *n*	Peptide No.	Antagonistic activity	Agonistic activity	Peptide No.	Antagonistic activity	Agonistic activity
2	2	4	19	−	−	4	−	−
2	3	5	20	+	−	5	−	−
2	4	6	21	+	+	6	NT[b]	±
3	2	5	22	+	−	7	−	−
3	3	6	23	−	−	8	−	−
3	4	7	24	−	−	9	−	−
4	2	6	25	+	−	10	NT	±
4	3	7	26	−	−	11	−	−
4	4	8	27	−	−	12	−	−
6	2	8	28	+	−	13	−	−
6	3	9	29	−	−	14	NT	+
6	4	10	30	−	−	15	−	−

Agonistic or antagonistic activities were considered positive only if the tested peptide exhibited over 50% stimulatory activity (compared with the activity obtained by the injection of 1 nmol of PBAN1–33NH_2 for 2 h) or an inhibitory activity (50% decrease in pheromone biosynthesis evoked by the injection of 0.5 pmol of PBAN1–33NH_2 for 2 h). Antagonistic activity of peptides was determined as described in the legend to Fig. 2. Agonistic activity was determined as described in the legend to Fig. 3. Antagonistic activity of peptides 6, 10, and 14 was not tested due to their agonistic activity.

[a] See Fig. 1, *A* and *B*.

[b] **NT**, not tested.

nous PBAN.[2] The use of cyclization in the design of antagonists with higher selectivity and metabolic stability has been applied successfully to other peptides such as oxytocin, glucagon, enkephalin, and luteinizing hormone-releasing hormone (for review, see Refs. 59 and 60, and references therein).

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 (62). The design of this peptide was based on computational studies of the C-terminal sequence of Lem-PK and exhibited 1 and 10% of the pheromonotropic agonistic activity of Lem-PK and Bom-PBAN, respectively (62).

Availability of selective agonists or antagonists is of major importance in the study of neuropeptides in general and for the study of the pyrokinin/PBAN family of neuropeptides in particular. This family of peptides is implicated in the regulation of critical reproductive, development, and digestive processes such as sex pheromone biosynthesis, cuticular melanization, myotropic activity, oviposition, pupariation, and diapause in moths and other insects (for review, see Ref. 11). Studies that were performed in several laboratories including ours have shown that PBAN and members of the pyrokinin family demonstrate considerable cross-activity (for review see Ref. 11). Despite the intensive studies of the bioactivity of this family of peptides, very little is known about the endogenous mechanisms and structural, chemical, and cellular basis of their activity. It is still not known which endogenous peptides mediate each of these functions *in vivo* and what are the characteristics of the receptor(s) that are involved in these processes. The BBC antagonists that were generated in this study will enable us to correlate between PBAN and its physiological functions in moths and other insects. These studies will reveal new functions mediated by the pyrokinin/PBAN family, disclose the presence of multiple pyrokinin/PBAN receptors and relate the exact physiological function to each peptide and receptor. The BBC peptides of the two sub-libraries described in this study have been tested, so far, only on their stimulatory or inhibitory activity of sex pheromone biosynthesis in female moths. Experiments on the ability of the BBC peptides from both sub-libraries to inhibit or stimulate other functions mediated by the pyrokinin/PBAN family are in progress. It is anticipated that once this study is completed, a series of selective and nonselective agonists and antagonists for *in vivo* studies will be available for the better understanding of the cellular and physiological basis of the pyrokinin/PBAN-mediated activities.

In summary, in this article we present a combined novel approach of rational design and selection method for the generation of agonistic and/or antagonistic cyclic peptides based on a linear lead peptide. This approach led to the discovery of several antagonists and agonists which exhibited either pheromonotropic activity or effectively inhibited sex pheromone biosynthesis in *H. peltigera* female moths. To the best of our knowledge, this is the first report on the use of backbone cyclization for the design of insect neuropeptide antagonists and the first report on the disclosure of PBAN pheromonotropic antagonists. Beyond the immediate benefits that are introduced by the cyclic peptides as selective antagonists, the information on the bioactive conformations of the antagonists that was gained in the course of this study may serve as a basis for the design of improved non-peptide mimetic agonists and antagonists. Such compounds are potential candidates for agrochemical applications, which can serve, after formulation and preliminary field experiments, as prototypes for the development of a novel group of highly effective, insect-specific and environmentally friendly insecticides.

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