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Coding genes and molecular structures of the diffusible signalling proteins (pheromones) of the polar ciliate, *Euplotes nobilii*

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ABSTRACT

In protozoan ciliates, diffusible signalling proteins (pheromones) regulate the vegetative growth and mating interactions. Here, the coding genes and the structures of the encoded pheromones were studied in genetically distinct wild-type strains representing interbreeding Antarctic and Arctic populations of the marine ciliate *Euplotes nobilii*. Determination of seven allelic pheromone-coding DNA sequences revealed that an unusual extension and high structural conservation of the 5′ non-coding region are peculiar traits of this gene family, implying that this region is directly involved in the mechanism of pheromone gene expression, possibly through phenomena of intron splicing and/or frame-shifting. For four pheromones, the three-dimensional structures were determined by nuclear magnetic resonance spectroscopy in solution. These structures show that the pheromones represent a protein family which adapts to its polar environment by combining a structurally stable core of a three-helix bundle with extended polypeptide segments that are devoid of regular secondary structures and concomitantly show enhanced structural flexibility.

1. Introduction

Protozoan ciliates represent one of the major micro-eukaryotic components of the polar ecosystems (Petz, 2005; Petz et al., 2007). They can be collected from practically every aquatic habitat and expanded with relative ease into stable laboratory cultures, which represent optimal material for studies of genes and proteins responsible for cold-adaptation in organisms that are at the same time individual eukaryotic cells directly exposed to the environment and natural selection.

Through several research trips to Antarctic, Fuegian and Arctic areas we had the opportunity to assemble a vast collection of wild-type strains representing a variety of marine species of the genus *Euplotes* (Di Giuseppe et al., 2011), which is the ciliate with the widest environmental distribution and diversity of species (Borror and Hill, 1995). The strains representing *E. nobilii* raised particular interest. Like other strains of *Euplotes* species inhabiting temperate waters, such as *E. patella*, *E. raikovi*, *E. octocarinatus* and *E. crassus*, they are capable of secreting cell type-specific signalling proteins that are genetically controlled at a single multi-allelic locus (designated as mating-type, or *mat* locus) (Dini and Nyberg, 1993). These water-borne proteins, usually known as pheromones, are functionally associated with the genetic mechanism of the mating types and act as prototypic autocrine (autologous) growth factors, as well as paracrine (heterologous) inducers of mating pair formation (Vallesi et al., 1995; Luporini et al., 2005). For a significant number of pheromones, it was possible to determine the full-length sequences of the coding genes expressed in the transcriptionally active genome of the cell somatic nucleus (macronucleus) (La Terza et al., 2009; Vallesi et al., 2009), as well as their molecular structures by NMR spectroscopy in solution (Pedrini et al., 2007; Placzek et al., 2007; Alimenti et al., 2009; Di Giuseppe et al., 2011). Comparative analysis of these genes and protein structures provides unique opportunities to derive a rather complete picture of their distinctive organization at the level of the homologous families of genes and proteins that they represent.

2. Origin of pheromones and structural characterization

Among the five *E. nobilii* strains covered by this study, two Antarctic strains (AC-1 and AC-4) were collected from coastal sites surrounding the Antarctic Italian Research Station at Terra Nova Bay (Ross Sea), and three Arctic strains (4Pyrm4, 2QAN1, and 5QAA15) were collected from coastal sites of Spitzbergen Island (Svalbard Archipelago) and the Qaanaaq-Thule area in Western Greenland (Di Giuseppe et al., 2011). In the laboratory, these strains were grown in cold rooms at 4 °C, using the green alga *Dunaliella tertiolecta* as the nutrient.

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The methods used for the determinations of the pheromone-gene sequences and pheromone NMR structures have been described in the original publications on these results (Pedrini et al., 2007; Placzek et al., 2007; Vallesi et al., 2009; Di Giuseppe et al., 2011).

3. Pheromone genes

The full-length sequences of seven distinct pheromone genes have been characterized. The three sequences *en-ant1*, *en-ant2* and *en-ant6* specify the pheromones E*n*-1, E*n*-2 and E*n*-6 from Antarctic strains (E*n*-1 and E*n*-2 from strain AC-1, E*n*-6 from strain AC-4). The four sequences *en-arc1*, *en-arc2*, *en-arc3* and *en-arc4* specify the pheromones E*n*-A1, E*n*-A2, E*n*-A3 and E*n*-A4 from Arctic strains (E*n*-A1 and E*n*-A4 from strains 4Pyrm4 and 2QAN1, respectively, E*n*-A2 and E*n*-A3 from the strain 5QAA15). The observation that some strains contain two distinct pheromone-gene sequences and co-secrete two distinct pheromones while other strains possess a single pheromone-gene sequence and secrete a single pheromone, is rationalized on the basis of previous studies of the mating-type inheritance in *Euplotes* (Heckmann and Kuhlmann, 1982; Luporini et al., 1986) by assuming that the former strains contain a heterozygous combination at the mating-type (*mat*) locus of their transcriptionally inert nucleus (the germinal "micronucleus", which generates the expressed macronuclear pheromone sequences), while the latter strains contain a homozygous combination.

The lengths of the seven DNA sequences vary from 900 (*en-ant1*) to 961 bp (*en-arc4*) excluding the telomeric inverted 5′-C_4A_4 and 3′-G_4T_4 repeats that cap the extremities of each sequence, and present the same basic structure with multiple start (ATG) and stop (TAA or TAG) codons (Fig. 1). However, only the strictly conserved start ATG codon found in position 362 of all sequences (361 in *en-ant6*) identifies an open reading frame (ORF) of 252 (*en-ant1*) to 285 (*en-ant6*) bp, which is specific for the pheromone synthesis in the form of cytoplasmic precursors (pre-pro-pheromones).

The 5′ and 3′ non-coding regions extend for nearly three-fold the length of the coding region. The 5′ region appears to be significantly more strictly conserved than the 3′ region (88 to 97% *vs.* 43 to 92% identity), in which the only functional element common to all the seven sequences appears to be a substitution of the conventional AATAAA polyadenylation signal with a TTATTT motif. In addition to showing a functional replacement of the conventional TATA or CAAT boxes for the initiation of transcription with the less conventional GAAAA motif (Ghosh et al., 1994), the 5′ region includes several consensus donor GT and acceptor AG splice-site junctions (Lescasse et al., 2005). This suggests the presence of one or more intron sequences destined to be alternatively removed, with the consequence that more than one mRNA species and hence more than a single pheromone isoform are synthesized.

4. Pheromone structures

The seven *E. nobilii* pheromones appear to be synthesized as "immature" cytoplasmic precursors (pre-pro-pheromones), as previously shown to be the case in *E. raikovi* (Miceli et al., 1991) and *E. octocarinatus* (Teckentrup et al., 1996). These *E. nobilii* pheromone precursors have chain lengths of 83 to 94 amino acid residues and are subject to proteolytic processing before the pheromones are secreted in their active form. A co-translational process removes a fully conserved pre-segment of 19 amino acids (i.e., Met-Thr-Lys-Leu-Ser-Ile-Phe-Val-Val/Met-Ile-Ala-Met-Leu-Val-Met-Val-Ser-Ser/Thr-Ala), and a post-translational cleavage removes a less strictly conserved pro-segment of 12 amino acids (i.e., Phe-Arg-Phe-Gln-Ser-Arg/Lys-Met-Lys/Arg/Asn-Ala-Lys/Gln-Thr-Glu/Gly/Ser/Ala). While the former putative cleavage (represented by an Ala-Phe dipeptide preceded by the combination Ser-Ser/Thr) involves a site that agrees well with consensus sequences of known signal peptides (Nielsen et al., 1997), the latter putative cleavage site (coincident with the combinations Gly-Asn/Asp, Ser-Thr, Ala-Tyr and Glu-Thr/Asp) appears to be unconventional for the enzymatic activities of known endopeptidases.

The secreted, active *E. nobilii* pheromones are characterized by amino acid sequences with levels of pairwise identity from 22 to 52% (Fig. 2). Strict conservation in the group of seven pheromones is limited to nine residues, eight of which are cysteines involved in the formation of four highly conserved disulfide bonds (Cys-I/Cys-VI, Cys-II/Cys-IV, Cys-III/Cys-VIII and Cys-V/Cys-VII) and one is an alanine residue. Nevertheless, the amino acid compositions are closely similar and overall reflect the adaptation of the *E. nobilii* pheromone family to cold environments. This adaptation is clearly apparent from a comparison with the amino acid composition of the pheromone family of an *Euplotes* species, *E. raikovi*, that lives in temperate sea waters and is phylogenetically closely related to *E. nobilii* (Vallesi et al., 2008).

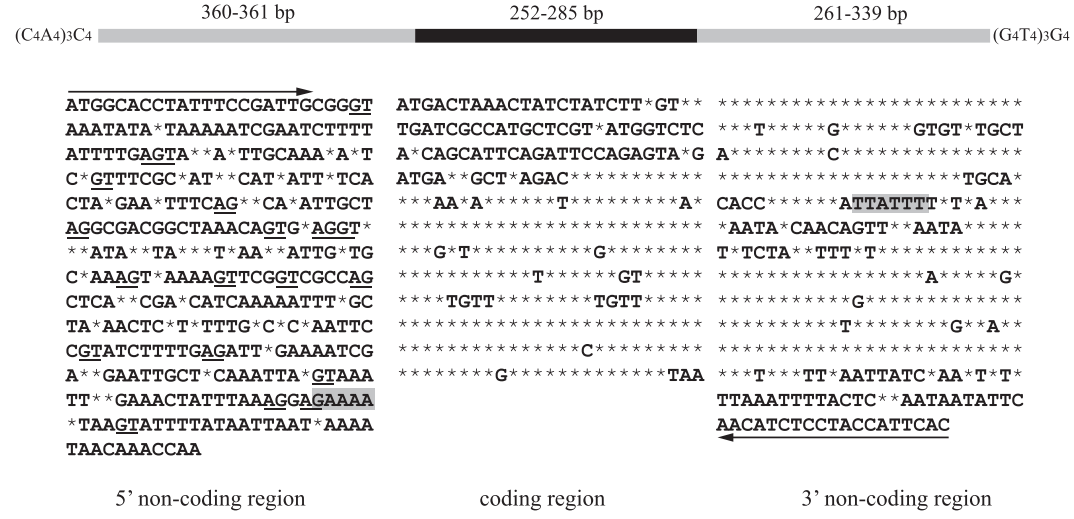

Fig. 1. Gene sequences coding for *E. nobilii* pheromones. Consensus was obtained by aligning seven sequences from the GenBank/EMBL databases (FJ645718, *en-ant1*; FJ645719, *en-ant2*; EF030059, *en-ant6*; FJ645720, *en-arc1*; FJ645721, *en-arc2*; FJ645722, *en-arc3*; FJ645723, *en-arc4*) with the Clustal W algorithm and optimizing this alignment through deliberate insertion of gaps. At the top, the overall gene organization is shown, with the telomeric ends characterized by inverted repeats, and gray and black segments indicating the relative extensions of the non-coding and coding regions. In the main body of the figure, which provides the gene sequence, bold capital letters identify fully conserved nucleotides and asterisks indicate variable positions (including inserted gaps). Donor and acceptor splice site junctions are underlined, putative signals for initiation of transcription and for polyadenylation are shadowed, and arrows indicate positions and directions of the oligonucleotide segments synthesized as PCR primers.

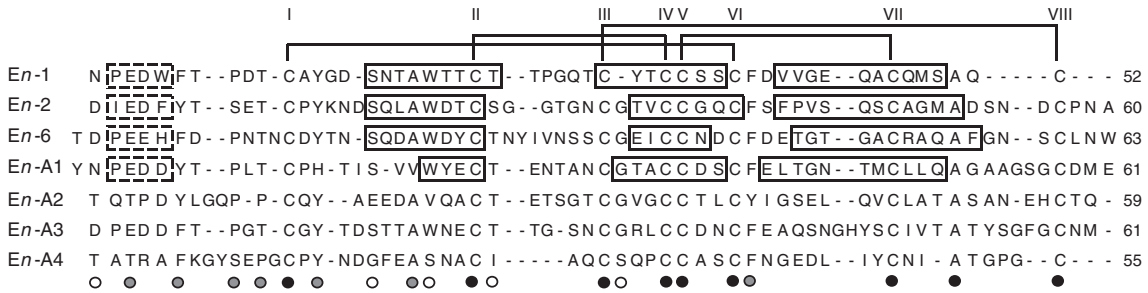

Fig. 2. Multiple amino acid sequence alignment of *E. nobilii* pheromones. The alignment was based on the Clustal W algorithm and optimized through deliberate insertion of gaps. Boxes delimited by a continuous line enclose residues involved in the formation of regular α-helices as determined in four pheromone NMR structures, while boxes delimited by a dashed line enclose residues involved in the formation of 3_{10}-helical turns. Cysteine residues are identified with Roman numbers progressing from the amino-terminus to the carboxy-terminus, and they are connected to show the disulfide pairings. Positions with absolute amino acid conservation are marked with black circles, and positions with high conservation, i.e. occupied by either two or three different amino acids, are marked with gray or empty circles, respectively.

While the two protein families show similar contents of charged amino acids (15%), the *E. nobilii* pheromones are significantly richer in polar and aromatic amino acids than the *E. raikovi* pheromones (44% *vs.* 33% and 11% *vs.* 6%, respectively) and, conversely, significantly poorer in hydrophobic amino acids (45% *vs.* 55%) (Table 1). Additional significant differences between the *E. nobilii* and *E. raikovi* pheromone families are observed for, (i) the content of amino acid residues located in α-helices (46.3% *vs.* 65.1%), (ii) the global hydrophilicity (−0.35 *vs.* −0.01) determined by the GRAVY index which is indicative of improved molecular interactions with the solvent (Schiffer and Dötsch, 1996), (iii) the global hydrophobicity (average values of 34.3 *vs.* 65.5) determined by the aliphatic index which manifests stabilizing hydrophobic forces in the protein core (Arnórsdottir et al., 2002), and (iv) the net charge (−9.5 *vs.* −7.1) calculated for a sea water pH value of 8.0.

Three-dimensional structures of the pheromones E*n*-1, E*n*-2 and E*n*-6 of Antarctic origin and the pheromone E*n*-A1 of Arctic origin, have been determined by homonuclear solution ^{1}H NMR analysis of purified protein preparations (Pedrini et al., 2007; Placzek et al., 2007; Alimenti et al., 2009; Di Giuseppe et al., 2011). They all show a core formed by an up–down–up three-helix bundle and a conserved pattern of four disulfide bonds, which is associated with three extended polypeptide segments with non-regular secondary structure (Fig. 3). In contrast to the three-helix core and the structurally disordered carboxy-terminal polypeptide segment, which have close counterparts in the *E. raikovi* pheromones, a well-defined amino-terminal segment of 14 to 17 residues and a loop of 5 to 10 residues between the first and second helices appear to be unique to the *E. nobilii* pheromone structures. Therefore, these two structural features are hypothesized to be cold-adaptive specificities that enhance the overall flexibility of the polypeptide backbone of *E. nobilii* pheromones. Support for this hypothesis comes from the locations of charged and aromatic amino acid side chains.

High density of negatively charged side chains is localized on the amino-terminal extensions of the *E. nobilii* pheromones (Fig. 3A). Such clusters of charged residues appear to be a common feature of the molecular surface of psychrophilic enzymes (Fedoy et al., 2007) and, due to their effect on widening the solvent-accessible areas and enhancing solvent interactions, they have usually been correlated with the cold-adaptive function of weakening the packing of the molecular structure (Lonhienne et al., 2000; Siddiqui and Cavicchioli, 2006). The polypeptide segment linking the first to the second helix neither carries exposed negative charges nor shows other apparent physicochemical specificities. However, its high content of Gly residues (four Gly in a total of six residues in the pheromone E*n*-2) and the polar residues Thr, Ser and Asn might contribute to locally enhance the flexibility of the polypeptide backbone by favoring interactions with the aqueous solvent.

The aromatic residues in the *E. nobilii* pheromone structures, which are twice as abundant as in the *E. raikovi* pheromones, may have a special structural role. Most of these residues occupy homologous positions in the different pheromones, mainly at the interface between the three-helix molecular core and the largely unstructured amino terminal extension (Fig. 3B). This positional conservation suggests that aromatic residues in the *E. nobilii* pheromones anchor the amino-terminal segment to the molecular core. However, the aromatic

Table 1
Amino acid composition of Antarctic and Arctic *E. nobilii* pheromones.

residues (%)	Antarctic				Arctic					Mean Arctic + Antartic	Mean of *E. raikovi* pheromones[h]
	E*n*-1	E*n*-2	E*n*-6	Mean	E*n*-A1	E*n*-A2	E*n*-A3	E*n*-A4	mean		
Polar[a]	46.1	45.0	42.8	44.6	39.3	45.9	49.2	40.1	43.6	44.1	33.1
Hydrophobic[b]	46.0	43.3	43.0	44.1	45.8	49.3	40.7	52.8	47.1	45.6	55.0
Charged[c]	11.5	15.0	20.6	15.7	16.4	13.6	16.3	12.7	14.7	15.2	15.9
Aromatic[d]	11.4	10.0	14.4	11.9	9.7	6.8	13.0	11.0	10.1	11.0	6.8
Located in α-helices	55.8	45.0	44.4	48.4	44.3	N.D.	N.D.	N.D.	44.3	46.3	65.1
GRAVY[e]	−0.36	−0.37	−0.74	−0.49	−0.13	−0.13	−0.49	−0.12	−0.22	−0.35	−0.01
Aliphatic index[f]	18.9	29.3	29.5	25.9	49.7	58.0	22.5	41.1	42.8	34.3	65.5
Net charge at pH 8.0[g]	−8.4	−9.4	−12.4	−10.1	−11.4	−9.4	−9.4	−5.5	−8.9	−9.5	−7.1

N.D., not determined.
[a] Asn, Gly, Gln, Ser, Thr, Tyr.
[b] Ala, Cys, His, Ile, Leu, Met, Phe, Pro, Tyr, Trp, Val.
[c] Arg, Lys, His, Asp, Glu.
[d] Tyr, Trp, Phe, His.
[e] Calculated as the ratio between the sum of hydropathy values of all residues and the total numbers of these residues.
[f] Defined as the relative volume that the amino acid lateral aliphatic chain occupies in a protein.
[g] Sea water pH.
[h] Pheromones: E*r*-1, E*r*-2, E*r*-10, E*r*-11, E*r*-22, and E*r*-23.

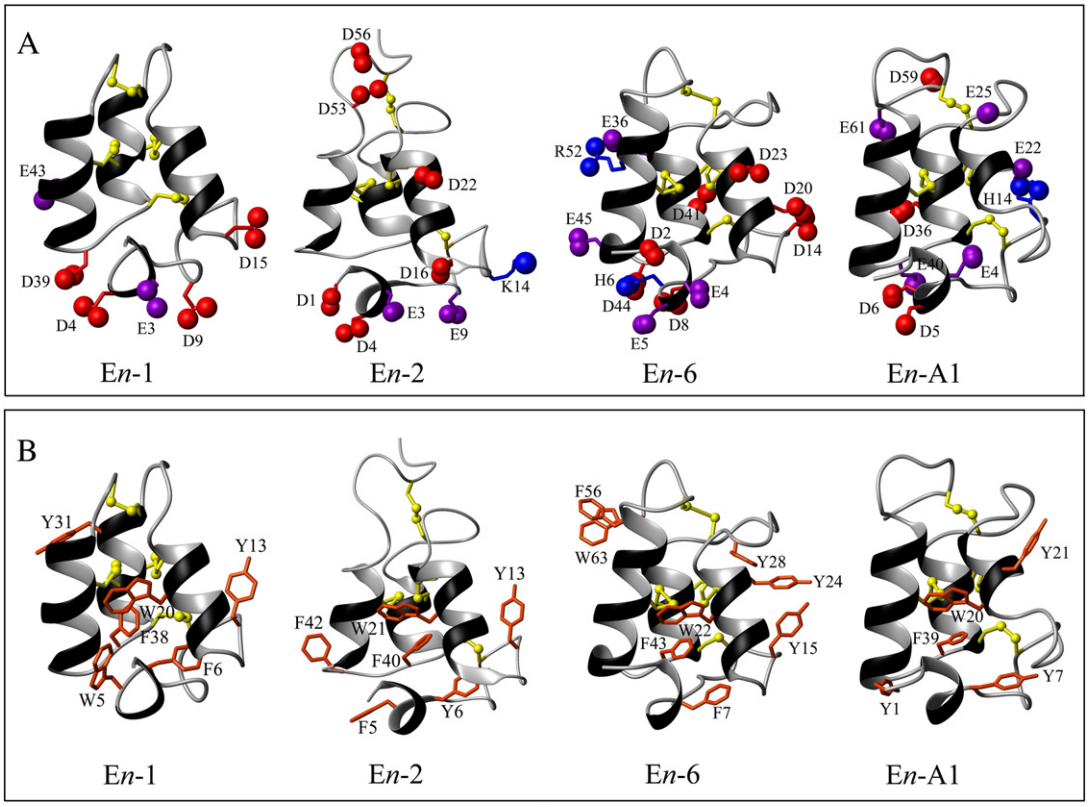

Fig. 3. Ribbon presentations of the NMR structures of four *E. nobilii* pheromones (Protein Data Bank deposits: 2NSV, E*n*-1; 2NSW, E*n*-2; 2JMS, E*n*-6; 2KK2, E*n*-A1). The disulfide bridges are shown as yellow-coloured stick diagrams. (A) Locations of electrostatically charged residues, which are identified with the one-letter amino acid code and the sequence position. Negative charges of Asp and Glu residues are represented by red and violet spheres, respectively, while positive charges of Lys, His and Arg residues are represented by blue spheres. (B) Locations of aromatic amino acid side chains, which are drawn as orange stick diagrams and identified with the one-letter amino acid code and the sequence position.

rings are not arranged in stacks or clusters, which is known to favor high stability of molecular structures (Burley and Petsko, 1985; Kannan and Vishveshwara, 2000). They rather interact with surrounding non-aromatic residues, forming hydrophobic contacts which are known to weaken at lower temperature (Kannan and Vishveshwara, 2000). The high of aromatic residues and their highly conserved positioning in the three-dimensional structures of the *E. nobilii* pheromones thus probably represents a functional compromise between the necessities of ensuring potential plasticity of the extended amino-terminal segment and maintaining this segment firmly anchored to the molecular core.

5. Conclusions

We determined the full-length nucleotide sequences of seven allelic genes coding for the *E. nobilii* pheromones, and the NMR solution structures of four of these diffusible signalling proteins. These results enable comparisons of genes and proteins that are members of the same homologous families, avoiding the phylogenetic noise that generally affects comparisons among individual genes and proteins of phylogentically distant organisms that are subject to different evolutionary pressures.

The comparison of the pheromone-gene sequences provides a major indication for the structure and function of the 5′ non-coding region. At odds with respect to the general organization of the gene-sized DNA molecules of the ciliate macronuclear genome (Jahn and Klobutcher, 2002; Prescott et al., 2002; Cavalcanti et al., 2004; Lescasse et al., 2005), this region exceeds the length of the coding region and shows high conservation, with a putative intron sequence and multiple potential TAA stop codons spaced in-frame with one another. All this strongly implies that, in addition to being functionally fundamental for the regulation of transcription, the 5′ region is itself a site of coding activity, possibly induced by mechanisms of alternative intron splicing and/or frame shifting. The former mechanism has already been described to take place in pheromone genes of other *Euplotes* species (Miceli et al., 1992; Brünen-Nieveler et al., 1998; Di Giuseppe et al., 2002), and frame-shifting appears to be a relatively common feature of *Euplotes* gene expression (Klobutcher and Farabaugh, 2002; Klobutcher, 2005; Vallesi et al., 2010). Additional observations on the mechanism of pheromone gene expression indicate that each *E. nobilii* pheromone gene synthesizes multiple mRNA's two of which would be compatible with the alternative removal of an intron sequence from the 5′ non-coding region (unpublished results).

The three-dimensional structures of *E. nobilii* pheromones show adaptive specificities that appear to be commonly implicated in the activity of psychrophilic proteins (Bae and Phillips, 2004). Chain elongation with polypeptide segments which are devoid of regular secondary structure appears to be the most direct way that *E. nobilii* pheromones have pursued to acquire a sufficient degree of flexibility of their molecular backbone to overcome the thermodynamically adverse conditions of a cold environment. This flexibility appears to be further enhanced locally by the presence of solvent-exposed clusters of negatively charged amino acid side chains, and by unique structural roles that aromatic residues take in *E. nobilii* pheromones at the interface of sub-domains in the molecular architectures.

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