

Biologically Active Orbitides from the Euphorbiaceae Family

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ABSTRACT

A comprehensive overview of natural orbitides isolated from Euphorbiaceae species and their most relevant biological activities are presented. Euphorbiaceae is a large and diverse family, which comprises about 300 genera, and is known as an important source of medicines and toxins. Several classes of secondary metabolites have been described for this taxon, however, orbitides have been broadly reported in *Jatropha* and *Croton* genera. Additionally, the latex is documented as the main source of orbitides in this family. Based on their structural and functional diversity, orbitides present a large variety of biological activities described as cytotoxicity, antimalarial, antibacterial, antifungal, enzymatic inhibition, and immunosuppressive, although the mechanism of action still needs to be further investigated. In recent years, the discovery of bioactive cyclic peptides from different sources has grown exponentially, making them promising molecules in the search for new drug leads. This review also highlights the attempts made by many researchers to organize the orbitides nomenclature and amino acid numbering, as well the important progress recently achieved in the biosynthetic study area.

ABBREVIATIONS

AMPs	antimicrobial peptides
CCK	cyclic cystine knot
CIP	Cahn-Ingold-Prelog
CPPs	cyclic peptides precursors
IUPAC	International Union of Pure and Applied Chemistry
OLP	oligopeptidase
PCY	peptide cyclase
PPIase	peptidyl-prolyl <i>cis-trans</i> isomerase
RiPPs	ribosomally synthesized and post-translationally modified peptides
SPPS	solid-phase peptide synthesis
UniProt	Universal Protein Resource

Introduction

Nature continues to guide researchers in the discovery and development of novel drug leads [1]. Cyclic peptides are structurally diverse and are found in all kingdoms of life. Traditionally, they have been found in animals [2], fungi [3], bacteria [4], and marine [5] sources, although higher plants have recently been shown to be a rich source of cyclic peptides with peculiar composition, stability, and structural and functional diversity [6, 7]. As a source of bioactive compounds, plants still play an important role in drug design and form the basis of many traditional medicines [1, 8]. Based on their structure, plant-derived cyclic peptides are classified into cyclotides [9–12], orbitides [7, 13], and sunflower-derived peptides [14]. Their pharmacological activities, applications, and structures have been extensively investigated [7, 15–19]. However, in addition to the obtained information, it is estimated that less than 1% of naturally occurring cyclic peptides have been discovered [20]. Orbitides constitute a class of natural cyclic peptides presenting a low molecular weight, ranging from 5 to 12 amino acids, ribosomally synthesized, with head-tail cyclization and a lack of

AMINO ACIDS

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Cys	cysteine
Glu	glutamic acid
Gln	glutamine
Gly	glycine
His	histidine
Ile	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine

disulfide bonds [17, 18, 21]. Cys is reported to be the least abundant amino acid observed in orbitides [17].

Angiosperm families are characterized by the biosynthesis of a diversity of classes of secondary metabolites. According to a literature survey, the orbitides are described only in the families Annonaceae, Caryophyllaceae, Euphorbiaceae, Lamiaceae, Linaceae, Phytolaccaceae, Rutaceae, Schisandraceae, and Verbenaceae [17].

The Euphorbiaceae family, comprising approximately 300 genera, is recognized due to its biosynthesizing of several classes of secondary metabolites, such as terpenoids, flavonoids, tannins, alkaloids, and steroids [22–24]. However, the members of this family are also described as a source of bioactive orbitides [13]. Even though Euphorbiaceae comprises most of the orbitides described in the literature, they were isolated from the genera *Jatropha* [25–34] and *Croton* [35–37]. The 1990s were considered the gold period of investigations of orbitides, with the discovery of several new molecules, thus demonstrating that the search for their mechanisms of action, structures, and biosynthesis needs to be expanded [7]. It should be noted that the majority of orbitides from Euphorbiaceae were isolated from the plant latex. Only a few compounds have been described in the leaves, stem bark, or aerial parts of the plant [25, 35].

In recent years, the discovery of bioactive cyclic peptides from different sources has grown exponentially, making them outstanding as the most promising molecules in the search for new drug leads [15, 16, 19, 38, 39]. The market for protein- and peptide-based drugs is over \$40 billion per year, and most of the available products are small peptides, ranging from 8 to 10 amino acid residues. These data reflect the number of papers found in the literature that primarily focus on peptide isolation, characterization, synthesis, pharmacological activities, and mechanism of action. It should be highlighted that over the years, more than 100 peptide

drugs have reached the market, demonstrating the pharmaceutical potential of these compounds [19].

In this review, we discuss the biological activities, chemical synthesis, elucidation techniques, classification, and biosynthesis of bioactive orbitides isolated from the Euphorbiaceae family. All information shown was gathered via searching English papers from scientific databases including PubMed, Elsevier, Google Scholar, Scopus, Web of Science, Cybase, and SciFinder by using the key words Euphorbiaceae, cyclic peptides, and orbitides.

Euphorbiaceae Family

The Spurge family (Euphorbiaceae) is one of the most complex, largest and most diverse families of angiosperms and occurs in arid and semiarid environments [40, 41]. It comprises approximately 300 genera and 8000 species, and it is established as an important source of medicines and toxins [42–44]. This family occurs mainly in the tropics, with the majority of the species in tropical and subtropical areas of Africa and the Americas. As might be expected from such a large and diverse family, there is a vast amount of information on its ethnomedical use, phytochemical investigations, and bioactive constituents. Williamson and Hooper [45] reported the use of *Euphorbia polycarpa*, *Euphorbia hirta*, and *Acalypha indica* L. for the treatment of different diseases in Ayurvedic medicine. In Chinese medicine, Lai and co-authors [46] reported 33 other species belonging to 17 genera of Euphorbiaceae used in herbal medicine. Among them, 17 species were used to treat snakebites, following the principle of “combat toxins with toxins”. Several species are grown as ornamental plants (*Euphorbia milli*, *Euphorbia tirucalli*, *Euphorbia obesa*, and *Euphorbia pulcherrima*), and many others are of considerable economic importance, such as cassava (*Manihot esculenta*), the castor oil plant (*Ricinus communis* L.), the rubber tree (*Hevea brasiliensis* Willd. Ex. A. Juss), and a biodiesel source (*Jatropha curcas* L.) [42]. As several species within the Euphorbiaceae family contain toxic compounds, some plants are usually described in the literature as poisonous, especially those from the *Jatropha* [43] and *Euphorbia* genera, which might induce dermatitis. Phorbol esters and curcins are described in the literature as the most toxic components found in *J. curcas* L. and exhibit toxicity to many species, including microorganisms, animals, and humans [47]. Many plants have oleaginous seeds, may contain latex, and provide a source of rubber [24, 40]. Latex is described as a complex mixture of peptides, enzymes, triterpenoids, diterpenoids, amino acids, and other compounds. Among the Euphorbiaceae family, latex is found primarily in members of the subfamilies Crotonoideae and Euphorbioideae, but is generally lacking in the Phyllanthoideae, Oldfieldioideae, and Acalyphoide subfamilies [24].

The Euphorbiaceae family presents a large variety of secondary metabolites, and the major types include alkaloids [23], triterpenoids [48, 49], flavonoids (both C- and O-glycosides) [49, 50], diterpenoids [23], tannins, and peptides (cyclic and linear) [13, 24, 48]. According to the literature, all orbitides isolated from Euphorbiaceae to date consist exclusively of L-amino acids. The D-isomers are not commonly available from natural sources and are usually obtained by synthetic approaches [51].

Classification

The IUPAC provides rigorous recommendations for the nomenclature of linear peptides, although many attempts have been made recently by several researchers to organize and clarify the orbitides nomenclature [20]. In 2006, Tan and Zhou [7] reviewed and classified a range of plant-derived cyclic peptides into two classes, five subclasses, and eight types based on their chemical structure and family origin. Initially, the classification of these small natural cyclic peptides as Caryophyllaceae-type cyclopeptides (type VI) was based on phytochemical studies of Caryophyllaceae species. It was believed that small cyclopeptides were characteristic components of Caryophyllaceae plants [7]. In general, this nomenclature was based on the botanical families, leading to an unclear generalization. As demonstrated, naming a group as “Caryophyllaceae-type” or “Rubiaceae-type” leads to the conclusion that all the peptides described in this botanical family occur exclusively in this taxon, which is not true [18]. Thus, in 2013, Arnison and co-authors [17] published a review on RiPPs and proposed the name “orbitide” to replace the older designation “Caryophyllaceae-type cyclopeptides”. In fact, RiPPs comprise a large group of natural peptides that are classified into subfamilies according to their biosynthetic pathway and the diversity of structural scaffolds. Caryophyllaceae-type homomonocyclopeptides, orbitides, and cyclotides are defined as RiPPs. In a general way, they can occur in many taxa and are genetically encoded and synthesized as precursor proteins [17, 52]. Additionally, according to Arnison and co-authors [17], it is too early to classify the various types of orbitides when only a few examples are reported in the literature. Recently, in 2015, Shim and co-authors [21], considering the disarray in the nomenclature of type VI cyclic peptides, proposed a new systematic naming system that reflects the taxonomic name of the species and a numbering system that enables systematic representation of the amino acid residues and modifications. According to the authors, this proposed nomenclature was based on IUPAC, UniProt, and the review published by Arnison and co-authors [17]. Following the instructions, the orbitide name should start with [1-#-N α C], which refers to the linkage between amino acid 1 and “#” through the α -amino group that is an N-C cyclization of the core peptide. Then add [#-Xaa, #-Xaa], which refers to the modifications and specifies the position and type of modified amino acid(s). A comma must be used to separate multiple post-translational modifications. In the sequence, add the taxonomic name abbreviation to identify the origin of the orbitide, which is based on the first three letters of the genus and the first two letters of the species name. Then add the common suffix (-orb) for orbitide. Finally, add the amino acid residue numbering (#) that begins at the N-terminal-most amino acid residue, based on the DNA sequence. As demonstrated in this review, papers published after this date (2015) describing the isolation of new orbitides started to adopt this nomenclature suggested by Shim and co-authors [21]. The use of this taxonomic nomenclature system allows the differentiation of orbitides with sequence diversity from closely related species.

Orbitides are described as small N-to-C terminal intramolecularly cyclized peptides from plants that do not have disulfide bonds, ranging from 5 to 12 amino acid residues. In contrast, cyclotides (type VIII) are natural peptides comprising 28–37 amino

acids, which are cyclized by both the fusion of the terminal amino and cysteine double bonds, thus containing three conserved disulfide bonds, known as a CCK [7, 9–11]. It was noticed that many amino acid residues might be underrepresented in orbitides. Cys is the least abundant amino acid observed, and some other residues such as Asp and Glu, their amides Asn and Gln, basic residues Lys and Arg, and His are also rare. Orbitides are found in higher plants but not in every taxon. So far, Annonaceae, Caryophyllaceae, Euphorbiaceae, Lamiaceae, Linaceae, Phytolaccaceae, Rutaceae, Schisandraceae, and Verbenaceae are the plant families described in the literature as containing orbitides [17].

Based on the discussion of cyclic peptide nomenclature and classification, a task group was recently designed by IUPAC to propose guidelines for homodetic cyclic peptides and specify amino acid priority for consistent numbering. IUPAC defines homodetic peptides as “cyclic peptides in which the rings consist only of amino acid residues in eupeptide linkage”. The amino acid numbering of peptides can be determined by various methods such as mass spectrum sequencing, alphabet, gene sequencing, and CIP rules. According to Craik and co-authors [20], the CIP system is unequivocal and can be generally applied to proteinogenic, nonproteinogenic, and unnatural amino acids. In the CIP rules, the priority of the amino acids is assigned by comparing the atom's atomic number. A table containing the priority ranking of the amino acids can be found in the mentioned paper [20], and according to this rule, proline takes the highest position, as it is the only amino acid that contains a secondary amine. The list of the 21 proteinogenic amino acids in the CIP priority is described as: Pro, Sec, Cys, Thr, Ser, Ile, Val, Met, Asp, Asn, His, Trp, Tyr, Phe, Leu, Glu, Gln, Arg, Lys, Ala, and Gly. Following this procedure, first identify the highest-ranking amino acid. Then, in case of duplicate amino acids, the next one on the C-terminal is compared. Finally, number the remaining amino acids from the N-terminal to C-terminal. Also, according to this rule, an *R* amino acid presents higher priority over its *S* isomer. As this system is not a formal IUPAC recommendation, the task group encourages further discussion and feedback from the scientific community [20]. In this review, compounds were numbered according to the CIP rules.

Biosynthesis

Understanding the biosynthetic mechanism is a challenge for all classes of peptides [53]. Not all of them are completely understood; sometimes the exact mechanism still remains unclear and needs to be further investigated. Regarding the biosynthesis of cyclic peptides from plants, the cyclotides present the best-understood pathway so far.

Based on their origins and biosynthetic pathways, in general, peptides can be organized into three groups: ribosomal, nonribosomal, and protein derivatives. Ribosomally synthesized peptides (RiPPs) are products of gene expression in which the amino acids are attached to one another during mRNA translation. The main classes of ribosomally synthesized cyclic plant peptides are the cyclotides, the SFTI-like peptides, also recognized as PawS-derived peptides (PDPs), and the orbitides [17].

Both orbitides and cyclotides differ greatly in arrangement and sequence [17, 54, 55]. Unlike cyclotides, orbitides have no disulfide bond, have a high proportion of hydrophobic residues, and

are processed in two steps involving two separate enzymes: cleavage by an oligopeptidase (OLP) and cyclization by a PCY defined as a serine protease-like enzyme [16, 54, 56]. According to some assays with seed extracts using synthetic peptide substrates, orbitides from *Saponaria vaccaria* are first ribosomally synthesized as linear precursors (32-amino acid linear precursor), termed pre-segetalin A1 [54, 57]. Further, biochemical assays were carried out in *S. vaccaria* seed extracts to determine the enzymes involved in cyclic peptide formation in Caryophyllaceae. The first enzyme from *S. vaccaria* is OLP1, a serine protease that recognizes and cleaves the sequence corresponding to the junction between the N-terminal region and the incipient cyclic peptide sequence. The OLP1 cleaves the first 15 amino acids to yield a product containing an N-terminal glycine (presegetalin A1 [14–32]) [56]. A second enzyme (PCY1) acts on this product to excise the linear C-terminal's 13 residues (presegetalin A1 [20–32]), with the concomitant macrocyclization of the remaining N-terminal's six residues to yield the c(Gly-Val-Pro-Val-Trp-Ala) segetalin A product. The biosynthesis of orbitides, as well as other RiPPs such as cyclotides, commonly uses convergent strategies based on Ser proteases that can catalyze the formation of a cyclic product via transamidation rather than a linear product via hydrolysis [56]. Biosynthetic studies were also conducted on the *Citrus* genus (Rutaceae), and the obtained data were consistent with the ribosome-dependent biosynthesis of orbitides via CPPs, which confirmed the role of the corresponding genes and provided evidence for the genes encoding CPPs [57].

Orbitides are generally derived from proteinogenic amino acids. Prior to 2011, very little was known about the biosynthesis of the orbitides and whether ribosomes were involved or not [57]. Recently, they started to be better understood once many aspects of RiPP biosynthesis and new insights were investigated [52]. The obtained data already described for systems such as Caryophyllaceae (*S. vaccaria*, *Dianthus caryophyllus*) and Rutaceae (*Citrus* spp.) are consistent and likely to provide further general insights into the mechanisms of enzyme activation and orbitide biosynthesis [57]. In 2011, the whole genome of *J. curcas* L. was sequenced, thus generating information for future research for this species and related plants. It was obtained using a strategy that combines the Sanger method and the new-generation multiplex sequencing methods. Also, when compared with genes from other plant species, it was indicated that 4% of genes are conserved specifically in the Euphorbiaceae family [58]. Finally, future efforts to obtain a better understanding of the biosynthetic pathway, genomic sequences, and the enzymes involved in RiPPs will contribute to the development and discovery of novel peptides with potential pharmaceutical and biotechnological applications [52, 54–56].

Peptides from the Croton Genus

Croton is a large genus from the Euphorbiaceae family comprising approximately 1300 species of trees, herbs, and shrubs commonly distributed in tropical and subtropical regions [23]. The seeds, roots, volatile oil, leaves, and stem bark are reported to contain several secondary metabolites [23] such as diterpenoids [59–62], triterpenoids, steroids, alkaloids, lignoids, proanthocyanidins, and flavonoids [37]. Some *Croton* species may contain latex, which is

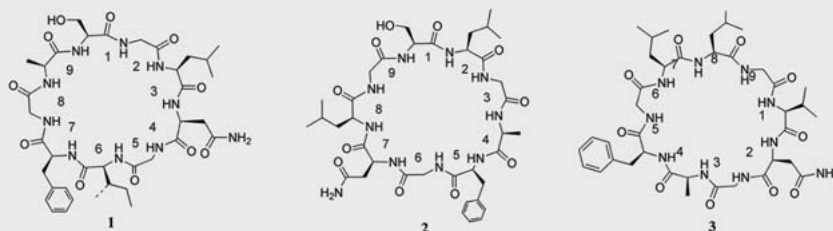
usually viscous and red colored [23]. Traditionally, the red sap, popularly known as dragon's blood or "sangre de gadro", is collected by cuts on the bark surface of several *Croton* species (*Croton lechleri*, *Croton palanostigma*, *Croton draconoides*, and *Croton urucurana* Baill.) and used by indigenous Amazon cultures and many others as a traditional medicine to promote wound healing [63, 64]. The *Croton* species are frequently associated with popular medicines for gastrointestinal ulcers and cancer, cicatrizants, and anti-inflammatory therapies [65, 66], and are reported to have a wide range of pharmacological properties such as mutagenic, apoptosis, antioxidant, antitumor, cytotoxic, and antiproliferative activity [23, 65–69]. Cyclic peptides (orbitides) [35–37] (Table S1, Supporting Information) and some linear peptides [22] isolated from the *Croton* species have been described in the literature.

Studies on the latex of *Croton gossypifolius* Vahl. led to the isolation of a cyclic nonapeptide named crotogossamide (1). This species was collected in Trinidad and Tobago but also occurs in Colombia, Venezuela, Mexico, and Martinique. Crotogossamide (1) was obtained as white crystals, and the amino acid sequence was determined to be *cyclo*(-Ser¹-Gly²-Leu³-Asn⁴-Gly⁵-Ile⁶-Phe⁷-Gly⁸-Ala⁹-) [37]. *C. urucurana* Baill. is one of the dragon's blood species mentioned above [36, 63, 64] and is commonly found in midwestern Brazil (Cerrado and Pantanal biomes). Examining the red latex of *C. urucurana* Baill. led to the isolation of an orbitide named [1–9-NaC]-crouorb A1 (2), a cyclic nonapeptide with an amino acid sequence defined as *cyclo*(-Ser¹-Leu²-Gly³-Ala⁴-Phe⁵-Gly⁶-Asn⁷-Leu⁸-Gly⁹-) [36]. The proposed orbitide name for this compound followed the systematic nomenclature recently suggested by Shim and co-authors [21]. From whole plant material of *Croton sparsiflorus* Morong, a shrub found in Asia and South America, a cyclic nonapeptide named crotosparsamide (3) was isolated (► Fig. 1). Crotosparsamide was obtained as white crystals, and the amino acid sequence was determined to be *cyclo*(-Val¹-Asn²-Gly³-Ala⁴-Phe⁵-Gly⁶-Leu⁷-Leu⁸-Gly⁹-) [35]. According to the literature data, all orbitides isolated from *Croton* species so far were defined as nonapeptides, and the absolute configurations of the amino acids were shown to be all L, thus highlighting the biosynthetic pathway.

In addition to the cyclic peptides already described, two linear peptides were isolated from the aerial parts of *Croton hieronymi* Griseb., a plant native to northwestern Argentina [22]. Compound 4 was described as the peptide derivative aurentiamide acetate, and according to Catalan and co-workers [22], it was previously found in *Aspergillus* species, two algae, and some higher plants. The other compound was a new linear peptide derivative named N-benzoyl-phenylalaninyl-N-benzoylphenyl-alaninate (5), which differed from compound 4 in that the left half was esterified by N-benzoyl-phenylalaninol and a second benzamide functionality was present. It should be highlighted that compounds of this type are rare and have not previously been found in *Croton* species [22] (► Fig. 2).

Peptides from the Jatropha Genus

The genus *Jatropha* comprises 170 species of woody trees, shrubs, and subshrubs distributed in tropical and subtropical countries. The name *Jatropha* derives from the Greek word *jatrós*, which means doctor, and the word *trophé*, which means food, thus implying the medicinal uses of these plants since ancient times [13,

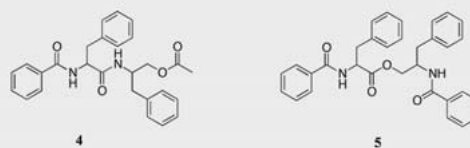


► **Fig. 1** The structures of orbitides (1–3) from *Croton* species.

70]. Currently, *Jatropha* species are widely used as medicinal plants in several countries from Asia, Latin America, and Africa, even considering the toxicity of different parts of the plant [13, 43, 71, 72]. They are commonly known for their popular uses for purgative, anti-allergic, wound healing, and anti-inflammatory effects [13]. Experimental studies described in the literature reveal antimicrobial, antineoplastic, wound healing, and hypotensive activities [13, 71]. In the past two decades, studies on *Jatropha* seed oil, especially *J. curcas* L. and *Jatropha gossypifolia* L., for biofuel have drawn attention, thus resulting in industrial-scale cultivation and making *Jatropha* species prospective high-quality biodiesel yielding crops [43, 71, 73].

Phytochemical studies on *Jatropha* plants led to the isolation of several chemical constituents [71, 72] such as alkaloids, triterpenoids, diterpenoids, sesquiterpenoids, flavonoids, coumarins, lignans, phorbol esters, phytosterols, and cyclic peptides. The *Jatropha* genus is characterized by the abundant production of latex, which is watery to white and contains saponin, resin, peptides, tannin, wax, and enzymes [70, 71]. A protease named curcain was obtained from the latex of *J. curcas* L. and reported to have wound healing activity in mice [70, 74]. The majority of cyclic peptides from *Jatropha* species were isolated from the latex, except for *Jatropha ribifolia* (Pohl) Baill. [25].

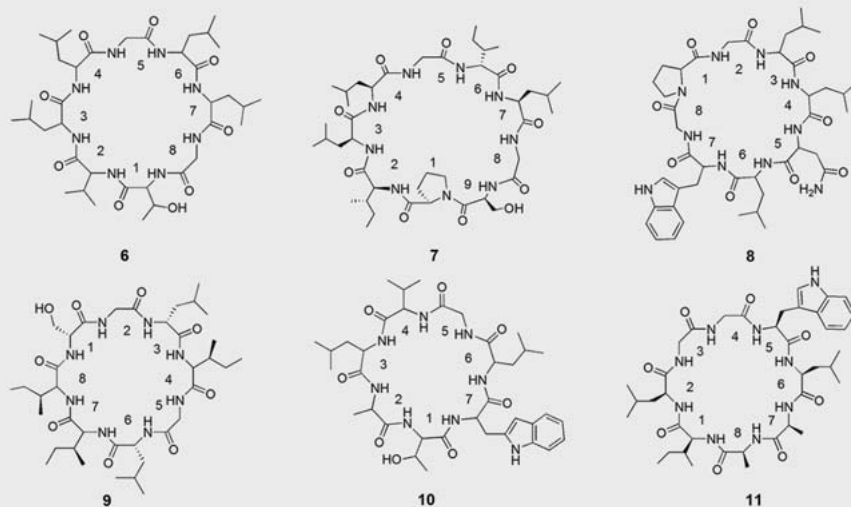
Investigations on the latex of *J. curcas* L., which is commonly known as a medicinal plant [70, 75], led to the isolation of three orbitides named curcacycline A (6) [29], curcacycline B (7) [30], and jatrophidin I [28] (8). Curcacycline A (6) is an octapeptide that was first isolated from an Indonesian plant, and its amino acid sequence was determined to be *cyclo*(-Thr¹-Val²-Leu³-Leu⁴-Gly⁵-Leu⁶-Leu⁷-Gly⁸-) [29]. The structure of curcacycline B (7), a nonapeptide, was determined to be *cyclo*(-Pro¹-Ile²-Leu³-Leu⁴-Gly⁵-Ile⁶-Leu⁷-Gly⁸-Ser⁹-), and the plant was obtained from Senegal [30]. Later, in 2012, Insanu and co-workers [76] applied a synthetic approach to confirm the structures of curcacycline A (6) and B (7), and also applied new pharmacological insights into these orbitides. Jatrophidin I (8) was isolated from a Brazilian plant, and the amino acid sequence was determined to be *cyclo*(-Pro¹-Gly²-Leu³-Leu⁴-Asn⁵-Leu⁶-Trp⁷-Gly⁸-). This compound contains an asparagine residue, which is not very common in cyclic peptides from plants. It was tested for some biological activities but was demonstrated to be inactive in antimalarial, antifungal, and antioxidant assays [28]. The cyclic octapeptide named ribifolin (9), an amorphous white solid, was isolated from the aerial parts of *J. ribifolia* (Pohl) Baill. This plant was collected in the semiarid northeastern



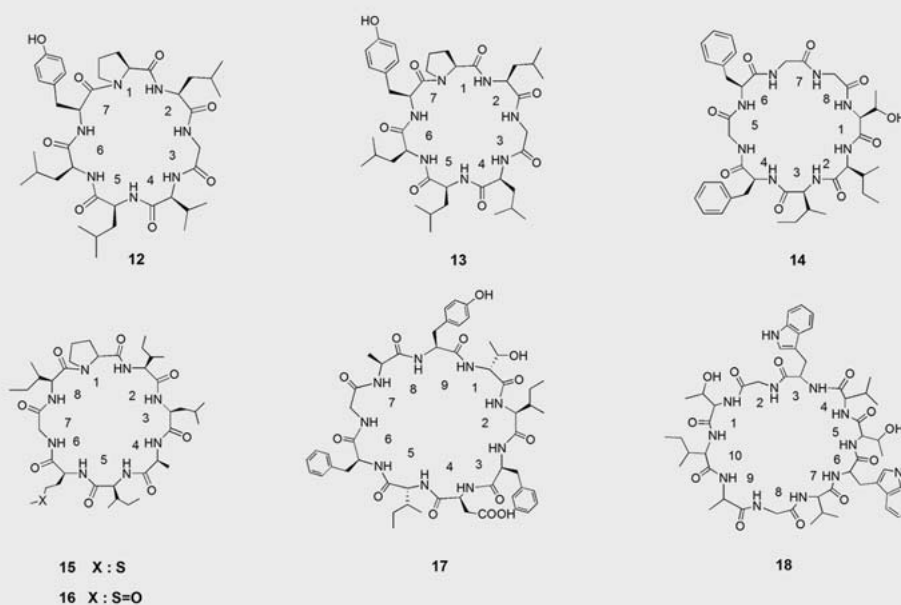
► **Fig. 2** The structures of linear peptides (4, 5) from *C. hieronymi* Griseb.

region of Brazil, and the sequence of the orbitide was determined to be *cyclo*(-Ser¹-Gly²-Leu³-Ile⁴-Gly⁵-Leu⁶-Ile⁷-Ile⁸-). Pinto and co-workers [25] also described the synthesis of the linear ribifolin analogue by an SPPS, which was used in further studies to determine the role of cyclization in some bioactivity assays. Studies on the latex of *J. gossypifolia* L. led to the isolation of two orbitides, one heptapeptide and one octapeptide, named cyclogossine A (10) [77] and cyclogossine B (11) [31], respectively. The structure of cyclogossine A (10) was determined to be *cyclo*(-Thr¹-Ala²-Leu³-Val⁴-Gly⁵-Leu⁶-Trp⁷-), and it was isolated from a plant collected in Indonesia [77]. Cyclogossine B (11) was isolated from a plant from Senegal, and the amino acid sequence was determined to be *cyclo*(-Ile¹-Leu²-Gly³-Gly⁴-Trp⁵-Leu⁶-Ala⁷-Ala⁸-) [31] (► **Fig. 3**).

A study on the latex of *Jatropha pohliana* ssp. *molissima* led to the isolation of two cyclic heptapeptides, pohlianins A (12) and B (13), and one cyclic octapeptide, pohlianin C (14) [26]. This plant was collected in the semiarid northeastern region of Brazil. The amino acid sequence of pohlianin A (12) was determined to be *cyclo*(-Pro¹-Leu²-Gly³-Val⁴-Leu⁵-Leu⁶-Try⁷-), the sequence of pohlianin B (13) was determined to be *cyclo*(-Pro¹-Leu²-Gly³-Leu⁴-Leu⁵-Leu⁶-Try⁷-), and the sequence of pohlianin C (14) was determined to be *cyclo*(-Thr¹-Ile²-Ile³-Phe⁴-Gly⁵-Phe⁶-Gly⁷-Gly⁸-) [26]. From the latex of *Jatropha chevalieri* Beille, a small shrub collected in Senegal, researchers obtained three orbitides named chevalierins A (15) and B (16), two cyclic octapeptides, and chevalierin C (17), a cyclic nonapeptide [34]. Locally, in folk medicine, the latex of this plant is used to treat infected wounds. The structure of chevalierin A (15) was determined to be *cyclo*(-Pro¹-Ile²-Leu³-Ala⁴-Ile⁵-Met⁶-Gly⁷-Ile⁸-), and the sequence of chevalierin C (17) was determined to be *cyclo*(-Thr¹-Ile²-Phe³-Asp⁴-Ile⁵-Phe⁶-Gly⁷-Ala⁸-Tyr⁹-). Chevalierin B (16) was found to be very similar and to have the same amino acid sequence as chevalierin A (15), and the only difference is the additional oxygen atom, suggesting the presence of methionine sulfox-



► **Fig. 3** The structures of orbitides (6–11) from *Jatropha* species.

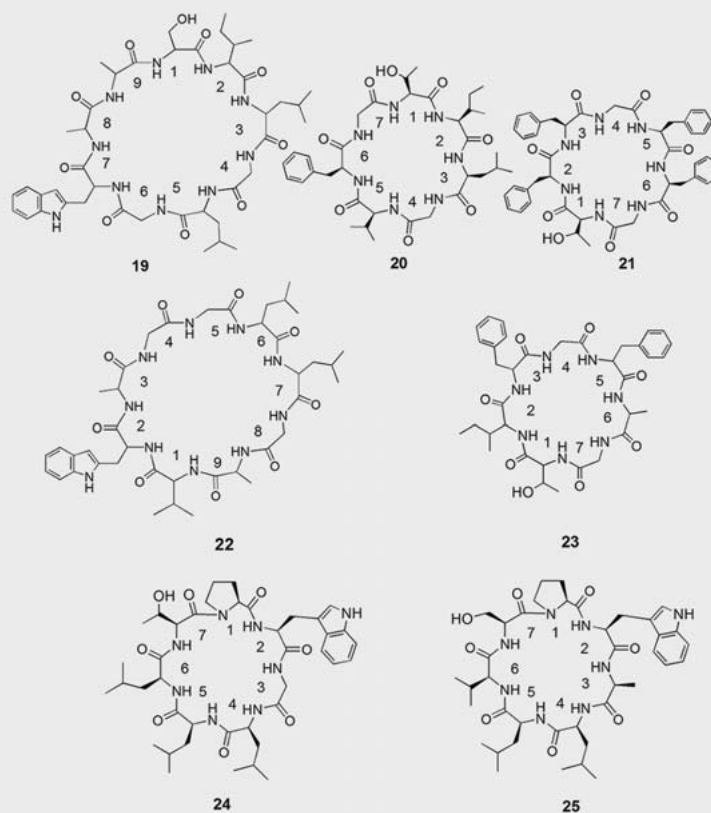


► **Fig. 4** The structures of orbitides (12–18) from *Jatropha* species.

ide (Mso) instead of methionine (Met). Thus, the structure of chevalierin B (**16**) was described as *cyclo*(-Pro¹-Ile²-Leu³-Ala⁴-Ile⁵-Mso⁶-Gly⁷-Ile⁸-). (► **Fig. 4**). They also described the synthesis of the chevalierins' linear precursors using a solid-phase technique [34].

From the latex of *Jatropha multifida* L. collected in Indonesia from leaf stalks, two orbitides, named labaditin (**18**) [32], a cyclic decapeptide, and biobollein (**19**) [78], a cyclic nonapeptide, have been reported. Its latex is commonly used in folk medicine for the treatment of infected wounds and skin infections. The structure of labaditin (**18**) was determined to be *cyclo*(-Thr¹-Ile²-Ala³-Gly⁴-Val⁵-Trp⁶-Thr⁷-Val⁸-Trp⁹-Gly¹⁰-) and according to Kosasi and co-authors [32], it was the first time that a cyclic decapeptide was

documented in higher plants. The sequence of biobollein (**19**) was determined to be *cyclo*(-Ser¹-Ile²-Leu³-Gly⁴-Leu⁵-Gly⁶-Trp⁷-Ala⁸-Ala⁹-) [78]. Examination of *Jatropha mahafalensis* Jum. & H. Perrier, a tree endemic to Madagascar and also used to treat infected wounds in traditional medicine, led to the isolation of two cyclic heptapeptides named mahafacyclin A (**20**) [79] and mahafacyclin B (**21**) [33]. The amino acid sequence of mahafacyclin A (**20**) was determined to be *cyclo*(-Thr¹-Ile²-Leu³-Gly⁴-Val⁵-Phe⁶-Gly⁷-), and according to the authors it was the first natural cyclic heptapeptide without proline to present the β -bulge characteristics [79]. It should be noted that the β -motif observed in cyclic heptapeptides usually contains at least one proline residue [26,



► **Fig. 5** The structures of orbitides (19–25) from *Jatropha* species.

33, 79]. Mahafacyclin B (21) was identified as a minor component of *J. mahafalensis* Jum. & H. Perrier latex, and the structure was determined to be *cyclo*(-Thr¹-Phe²-Phe³-Gly⁴-Phe⁵-Phe⁶-Gly⁷-), highlighted by the presence of four phenylalanine residues. The linear precursor of mahafacyclin B (21) was synthesized by a solution phase method [33]. Research on the latex of *Jatropha podagrica* Hook, a plant collected in Kenya, led to the isolation of two orbitides, podacycline A (22), a cyclic nonapeptide, and podacycline B (23), a cyclic heptapeptide. The structure of podacycline A (22) was determined to be *cyclo*(-Val¹-Trp²-Ala³-Gly⁴-Gly⁵-Leu⁶-Leu⁷-Gly⁸-Ala⁹-), and the structure of podacycline B (23) was determined to be *cyclo*(-Thr¹-Ile²-Phe³-Gly⁴-Phe⁵-Ala⁶-Gly⁷-) [27]. *Jatropha integerrima* Jacq. was collected in Thailand, and two cyclic heptapeptides, integerrimides A (24) and B (25), were isolated from the latex. The orbitide integerrimide A (24) was obtained as a colorless solid and identified as *cyclo*(-Pro¹-Trp²-Gly³-Leu⁴-Leu⁵-Leu⁶-Thr⁷-). Integerrimide B (25) was obtained as colorless needles and identified as *cyclo*(-Pro¹-Trp²-Ala³-Leu⁴-Leu⁵-Val⁶-Ser⁷-) [80] (► **Fig. 5**).

In addition to the presence of cyclic peptides in the latex of *J. curcas* L., an investigation of the seeds led to the isolation of a new linear peptide named PII-C, consisting of 14 amino acid residues and having a molecular mass of 1341.58 Da [M + H]⁺. The amino acid sequence was found to be Ala-Pro-Thr-Leu-Ser-Gly-Gly-Ser-Val-Pro-Arg-Asp-Ala-Asp [81].

It should be mentioned that all orbitides already described in the literature from *Jatropha* species have an L-configuration, a low amino acid complexity, and a high proportion of hydrophobic amino acids, which is commonly noticed in cyclic peptides isolated from the Caryophyllaceae group (Table S1, Supporting Information) [17, 18]. Concerning cyclic peptides, we can assume that the *Jatropha* genus has been investigated in greater detail than the other representative genera of the Euphorbiaceae family.

Peptides from Other Genera (Euphorbiaceae Family)

In addition to the orbitides predominantly described in *Croton* and *Jatropha* species, some other genera from the Euphorbiaceae family have also been identified as sources of peptides and derivatives. Those peptides are usually differentiated from orbitides by the presence of disulfide bonds, a lack of head-to-tail cyclization, and a linear amino acid sequence.

Ricinus communis L. is a plant from the Euphorbiaceae family known for the presence of the protein ricin, an extremely toxic natural poison found in high amounts in the plant. Ricin can be toxic by ingestion, inhalation, or injection [82, 83]. Recently, three new peptides, designated *R. communis* L. biomarkers (RCB) 1–3, were identified in the plant seeds. Those peptides comprise homologous sequences of 19 (RCB-1) or 18 (RCB-2 and RCB-3)

amino acid residues, including four cysteine moieties and disulfide bonds [82]. Thus, RCB-1 consists of 19 amino acids and has two disulfide bonds, and the structure was determined to be (-Ala-Arg-Cys-Cys-Leu-Val-Met-Pro-Val-Pro-Pro-Phe-Ala-Cys-Val-Lys-Phe-Cys-Ser-) [83]. It was synthesized by SPPS with the assistance of microwave heating during the deprotection and coupling reaction steps, and then subjected to oxidative folding. Additionally, the three-dimensional structure of RCB-1 was determined [82]. RCB-2 contains 18 amino acids and two disulfide bonds, and the sequence was determined to be (-Ala-Arg-Cys-Cys-Leu-Val-Met-Pro-Val-Pro-Pro-Phe-Ala-Cys-Val-Lys-Phe-Cys-). The peptide RCB-3 also has two disulfide bonds and has the sequence (-Ala-Arg-Cys-Cys-Leu-Val-Leu-Pro-Val-Pro-Pro-Phe-Ala-Cys-Val-Lys-Phe-Cys-) [83]. Due to their similarity to the Ib-AMP peptide family, it was suggested that the function of these RCB peptides might be related to plant defence, playing a role in seed protection, although further investigations are still suggested [82, 83]. The genus *Euphorbia* also contains peptides. A molecule identified as a peptide derivative, aurentiamide acetate, compound 4, was isolated from the roots of *Euphorbia fischeriana* Steudel [84]. As mentioned previously, this compound was also found in the aerial parts of *C. hieronymi* Griseb [22].

Biological Activities

Cytotoxicity

Cytotoxicity is a well-documented activity for orbitides. As found in the literature, some orbitides are cytotoxic, and others are demonstrated to be nontoxic to the cells. This different behavior may be related to their conformation, their sequence, the presence of charges, the number of amino acid residues, and the type of tumor cells involved. Thus, the mechanism of action needs to be further investigated. The orbitide [1–9-NaC]-crouorb A1 (2) isolated from the latex of *C. urucurana* Baill. was evaluated against six human cancer cell lines, namely, 786–0 (human kidney carcinoma), HT-29 (human colon carcinoma), MCF-7 (breast adenocarcinoma), NCI-ADR/RES (ovary adenocarcinoma, multidrug-resistance phenotype), PC-03 (human prostate carcinoma), and Hep-G2 (hepatocellular carcinoma). Compound 2 demonstrated selective inhibition against NCI-ADR/RES cells ($GI_{50} = 4.8 \mu\text{M}$) although according to the authors, further studies are necessary to determine the effects on nontumour cells [36]. The orbitide ribifolin (9), isolated from the aerial parts of *J. ribifolia* (Pohl) Baill., and its synthetic linear analogue were assessed for their cytotoxicity towards human embryonic kidney cells (HEK293T). No cytotoxicity was observed; normal growth occurred in the tested concentration range (0.001–100 μM) [25]. Cytotoxicity testing of curcacyclines, isolated from *J. curcas* L., demonstrated that curcacycline A (6) presented low toxicity to OVCAR 3 (ovarium cancer cells) with an IC_{50} of 945 μM , while no effect was found on Colo205 (human colon cancer cells). Curcacycline B (7) did not demonstrate any effect on either cell line. Additionally, they were evaluated in a brine shrimp cytotoxicity assay, and curcacycline A (6) and B (7) showed LD_{50} values of 65.07 μM and 18.35 μM , respectively [76].

Antimalarial

The orbitide ribifolin (9) and its linear synthetic analogue were evaluated for antimalarial activity. Ribifolin (9) demonstrated moderate activity with an IC_{50} of 42 μM against the *Plasmodium falciparum* 3D7 strain, while the linear analogue was inactive ($IC_{50} = 519 \mu\text{M}$), providing evidence that cyclization is important for the growth inhibitory activity against *P. falciparum* [25]. The pohlianins, isolated from *J. pohliana* ssp. *molissima*, were also evaluated for antimalarial activity, and moderate inhibition was obtained for these compounds. Pohlianin A (12) and B (13) had IC_{50} values of 57 μM and 25 μM , respectively. Pohlianin C (14) showed an IC_{50} of 16 μM and was the most potent among the pohlianins [26]. The three chevalierins A (15), B (16), and C (17), obtained from *J. chevalieri*, were examined for antiproliferative activity against *P. falciparum*, but only chevalierin A (15) showed antimalarial activity, with an IC_{50} of 8.9 μM [34]. The mahafacyclins, obtained from *J. mahafalensis* Jum. & H. Perrier [33, 79], were evaluated for antimalarial activity. Mahafacyclin A (20) presented a moderate antimalarial activity with an IC_{50} value of 16 μM [79]. Mahafacyclin B (21) was the most potent and possessed an IC_{50} of 2.2 μM *P. falciparum* [33]. Integerrimides A (24) and B (25), from the latex of *J. integerrima* Jacq., were evaluated in antimalarial assays but demonstrated to be inactive at test concentrations of 12.8 μM and 13.0 μM , respectively [80]. Jatrophidin I (8) was also inactive in antimalarial assays [28].

Immunosuppressive

Autoimmune diseases can be classified as organ-specific or as systemic. On a molecular level, autoreactive T cells play an important role in disease progression [85]. The orbitide curcacycline A (6), isolated from *J. curcas* L., showed a moderate dose-dependent inhibition of human T cell proliferation, although direct cytotoxic effects could not be observed [29]. Also, an immunologically active peptide, labaditin, was isolated from the latex of *J. multifida* L. [32]. Usually, the immunosuppressive activity of orbitides is characterized by weak affinity to their molecular target, calcinerium. Other members of the orbitide family, such as cyclolinopeptides, cycloleonorinins, and hymenistatins, also exhibited potential immunosuppressive activity [85].

Enzymatic Evaluation

Jatrophidin I (8), isolated from *J. curcas* L., showed inhibitory activity in a fluorimetric protease inhibition assay using pepsin as a molecular model for aspartic protease inhibition. Its IC_{50} value was 0.88 μM , although it did not inhibit the serine protease subtilisin, thus suggesting a selective inhibition for aspartic proteases [28]. According to the authors, the structure of curcacycline B (7) was suggested to be a PPlase substrate. PPlase assays using human cyclophilin B, based on an α -chymotrypsin-rotamase coupled enzymatic experiment, were carried out to determine if curcacycline B (7) could bind human cyclophilin B. Curcacycline B (7) was shown to enhance the rotamase activity of human cyclophilin B. PPlase assays using human cyclophilin B were carried out to determine if curcacycline B (7) could bind the human cyclophilin B. In the absence of cyclophilin B, curcacycline B (7) had no effect on α -chymotrypsin activity, although when the experiment was carried out

in the presence of cyclophilin B, it enhanced the PPLase activity of cyclophilin B by 60% at 30 μM .

Antibacterial and antifungal

The search for new classes of antibiotics, such as AMPs, has become even more relevant as the resistance of bacteria has reached higher levels worldwide. Investigation of the antibacterial activity of labaditin (**18**), isolated from *J. multifida* L., demonstrated a reduction of viability in gram-positive bacteria (*Streptococcus mutans*), while its linear analogue is not active, thus demonstrating that cyclization might contribute to the antibacterial activity. No effects were observed in gram-negative bacteria (*Aggregatibacter actinomycetemcomitans*) for concentrations ranging up to 100 mM [86]. Labaditin (**18**) and its linear analogue were also evaluated against *Staphylococcus aureus*. It was shown that the cyclic compound (**18**) was highly effective against *S. aureus*, with no bacterial growth at a concentration as low as 0.23 mM. On the other hand, the linear analogue required concentrations above 28.6 mM to be active. According to Barbosa and co-authors [87], the antibacterial activity of labaditin (**18**) was related to its inducing leakage in the cell membrane. Leakage occurs with the self-assembly of labaditin (**18**) into a nanotube owing to its preserved secondary structure in the membrane. Additionally, this mode of action may represent a promising step towards the design of a new class of antibiotics. The screening of curcacycline A (**6**) for antibiotic activity was done against five strains, *Bacillus subtilis*, *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*, using the paper disk (6 mm of diameter) diffusion method at a concentration of 1.3 mM. Curcacycline A (**6**) inhibited the growth of *B. subtilis* and *P. aeruginosa* with inhibition zones of (10.3 \pm 0.4 mm) and (8.3 \pm 0.8 mm), respectively. Although the mechanistic action of curcacycline A (**6**) still remains unclear, the antibacterial activity of cyclic peptides might be related to their ability to cross membranes and their higher affinity to bind with some proteins [76]. The orbitides integerrimides A (**24**) and B (**25**), obtained from the latex of *J. integerrima* Jacq., were evaluated for antifungal assays, although they were demonstrated to be inactive at concentrations of 64.1 μM and 65.3 μM , respectively [80]. In addition, jatrophidin I (**8**) was inactive in antifungal assays [28]. The antibacterial and antifungal activity of peptide RCB-1, isolated from *R. communis* L., was tested against human and plant pathogens. Different concentrations of RCB-1, ranging from 160 to 0.625 μM , were used. The compound RCB-1 was found to be active against only *P. aeruginosa*, although it was tested against a set of human pathogens (*E. coli*, *S. aureus*, and *C. albicans*) and plant pathogens (*Erwinia carotovora*, *Erwinia amylovora*, and *Aspergillus fumigatus*) [82].

Other Families as Orbitide Sources

Plants in different angiosperm families are notorious for accumulating peptides, primarily cyclic peptides [7]. According to the literature, in addition to being found in the Euphorbiaceae family, orbitides can also be isolated from at least eight other plant families, including Annonaceae, Caryophyllaceae, Lamiaceae, Linaceae, Phytolaccaceae, Rutaceae, Schisandraceae, and Verbenaceae [17, 18].

In fact, the orbitides can be isolated not only from plant latex but also from seeds, leaves, roots, twigs, and stem bark. The Rutaceae family is commonly known as the citrus family, which is also the most economically important genus. Studies on *Citrus* species [88–91] led to the isolation of orbitides, and they also might be found in the *Zanthoxylum* [92, 93], *Evodia* [94], and *Clausena* genera [95, 96]. Recently, in 2016, the orbitide named [1–8-N α C]-zanthoxylidin A1 was isolated from the leaves of *Zanthoxylum riedelianum* Engl. (Rutaceae) [93]. The proposed orbitide name of this compound followed the systematic nomenclature recently suggested by Shim and co-authors [21]. This compound induced cell death by apoptosis in Jurkat leukemia T cells with an IC₅₀ of 218 nM [93].

The Linaceae family is also a good source of orbitides. In 1959, the first orbitide, a cyclic nonapeptide from the flaxseed oil of *Linum usitatissimum* L. (Linaceae), was discovered by Kaufman and Tobschirble and named cyclolinopeptide A, cyclo(-Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile-) [97]. This cyclic nonapeptide exhibits several biological activities, and later on many additional cyclic peptides were isolated from linseed (cyclolinopeptide B–E and cyclolinopeptide F–I) [98, 99]. Actually, the cyclolinopeptides are assigned in the literature as significant compounds found in flaxseed, but like many other orbitides, the role of these molecules in the plants remains unknown [100]. The Annonaceae family comprises approximately 130 genera, but most of the orbitides documented in the literature are isolated from the genus *Annona* (*Annona squamosa*, *Annona cherimola*, *Annona glauca*) [101–103]. Caryophyllaceae is a family of flowering plants with 81 genera, commonly called the pink family. Some plants belonging to the Caryophyllaceae family contain cyclic peptides, and they are most commonly found in the genera *Drymaria* [104], *Dianthus* [105], *Pseudostellaria* [106–108], *Stellaria* [109–111], and *Vaccaria* [112].

In summary, more than 100 orbitides with various bioactivities have been discovered, and this number continues to grow as natural products remain an important source of bioactive compounds over the years [53]. Additionally, other classes of peptides might emerge with new bioactivities and novel applications, thus attracting the attention of many researchers worldwide.

Elucidation Techniques and Chemical Synthesis

Orbitide structures are mainly identified by two techniques, NMR and MS, which are very well established in this research field. In NMR spectroscopy, each resonance must be associated with a specific nucleus in the investigated molecule, a process called assignment. Thus, the data are assigned by one-dimensional (1D) ¹H, ¹³C, and ¹⁵N and two-dimensional (2D) techniques such as COSY, TOCSY, NOESY, and ROESY (rotating frame Overhauser effect spectroscopy) NMR. Several overlaps may occur in ¹H and ¹³C NMR spectra due to the similarity of the chemical shifts from different amino acid residues, which is the reason 2D NMR techniques became very suitable for structure determination [51, 113, 114]. In general, assignment can be done using homonuclear spectra in two steps: a) the sequential assignment of the amino acids in the peptide sequence and b) the assignment of the amino acid side chains [115].

Using TOCSY and COSY, it is possible to establish the identification of certain amino acid spin systems, with a characteristic pattern of cross signals. For example, Gly is the only residue in which two protons interact with the amide proton. Val, Leu, and Ile residues can be recognized by their two methyl groups, which give a characteristic row of double signals. In contrast, His, Trp, Tyr, and Phe residues are difficult to distinguish because $H\alpha$ interacts with two methylene protons that do not show any J coupling to any of the ring protons (more than three bonds removed). Therefore, these residues are also difficult to distinguish from Asp, Asn, Cys, and Ser residues [115, 116]. The next step in the assignment procedure concerns the identification of the sequence-specific position of each amino acid by a NOESY experiment. Briefly, the spectra obtained through this experiment are used to sequentially connect the spin systems. The connection of an amino acid in the sequence (i) to its following one ($i + 1$) can be observed in the NOESY because the distance of the amide proton of ($i + 1$) to the $H\alpha$, $H\beta$, or $H\gamma$ protons of (i) is frequently smaller than 5 Å. An exception is proline residues, which have no amide proton and for which no HN(i)- $H\alpha$ ($i-1$) cross signal can be observed. However, if the proline (i) is in its *trans* conformation, sequential HN($i-1$)- $H\delta$ (i) and $H\alpha$ ($i-1$)- $H\delta$ (i) cross signals can be observed [116].

MS measures the mass-to-charge ratio (m/z) of gas-phase ions and is important for the accurate mass determination and characterization of peptides, only requiring small amounts of the compounds [117]. As it should be performed in the positive mode, it shows the protonated molecule MH^+ and frequently some adduct ions $[M + Na]^+$ or $[M + K]^+$ in the MS spectrum. The most used method for the ionization of orbitides is electrospray ionization (ESI). ESI is a solution technique used in conjunction with mass analyzers such as tandem mass spectrometry. MS is very sensitive and does not require peptides to be purified. Finally, the MS data must be consistent with the amino acid sequence derived from the NMR experiments. It was noticed that peptide amino acid sequences are typically reported in the literature in the order in which they occur in MS/MS fragmentation [21]. In addition, using NMR studies and X-ray crystallography, it is possible to assign the 3D structures of the cyclic peptides, thus providing more detailed information on the peptide structure [51, 113]. NMR has the advantage that it can be done in solution and has proven to be a robust and widely applied technique [113]. In addition, crystallography gives the static structure of the molecule but requires the production of crystals, which are not often easily obtained in orbitides. Finally, the structures of orbitides have been proposed based on extensive 2D NMR investigation and HRMS [51].

The accessibility of orbitides by chemical synthesis has facilitated studies of their mechanism and activities, usually once just a few milligrams are obtained from the natural sources. The synthesis of cyclic peptides can occur in two different ways, in solution (classical synthesis) or in the presence of a polymeric support. The latter method is known as SPPS. SPPS is the most commonly used strategy due to its practicality, its higher speed, and the possibility of automation [114]. It is well established and applicable to any peptide synthesis. Thus, SPPS is considered a useful tool that can be applied to obtain cyclic peptide libraries and assist in biological assays, especially *in vivo* assays, which usually require higher amounts of compounds. Cyclic peptides differ from linear pep-

tides in that their backbones have one additional peptide bond that joins the N- and C-terminal to make a circular backbone [6]. The cyclization of linear peptides can be achieved in different ways, including head-to-tail, side chain-to-side chain, or terminus-to-side chain linkages, and, as was mentioned before, the orbitides are obtained through head-to-tail cyclization [118]. The synthesis of peptides containing a cyclic backbone is achieved by SPPS based on the 9-fluorenylmethoxycarbonyl (Fmoc) amino protection strategy of an acyclic (linear) precursor followed by an intramolecular reaction linking the C- and N-termini. Thus, the linear chain is cyclized via amide bond formation [119]. SPPS was first developed by Merrifield [120, 121] and still remains an efficient strategy to obtain peptide libraries with chemodiversity. Furthermore, this technique provided great improvement in the drug discovery of peptide mimetics, especially ones involving changes in the backbones of novel molecules containing unnatural amino acids, mutations, and chemical modifications, which might help to drive their application as drug scaffolds.

Conclusions

In the current review, we have demonstrated that natural products play an important role in pharmaceutical and biological research, in which peptide-based drugs have a prominent place due to their chemical versatility, stability, and specificity. Although the description of plant-derived cyclic peptides has increased in recent years, the mechanisms of action of only a limited number of compounds have been reported. Additionally, despite the increasing number of compounds over the years, it has become clear that orbitides are still underexplored. Many cyclic peptides found in Euphorbiaceae and other plant families await discovery and further investigation. In addition, according to the literature, only the *Jatropha* and *Croton* genera have been documented as sources of orbitides in the Euphorbiaceae family. As demonstrated, there are still many species that have received little to no attention. Lastly, to address the disarray of orbitides nomenclature, which has been classified three times in the past few years, much effort was devoted by researchers and improvement was obtained in the classification and numbering of orbitides, highlighting the importance of this class of compounds in higher plants.

Supporting information

A table showing additional information on orbitide sequences, molecular weight, plant species, and synonyms is available as Supporting Information.

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Conflict of Interest

The authors declare no conflicts of interest.

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