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Graphical abstract

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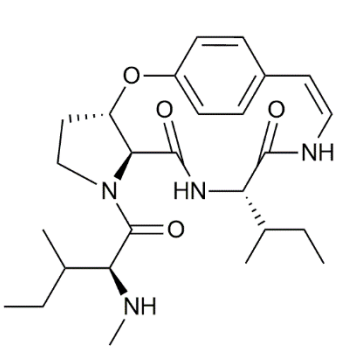
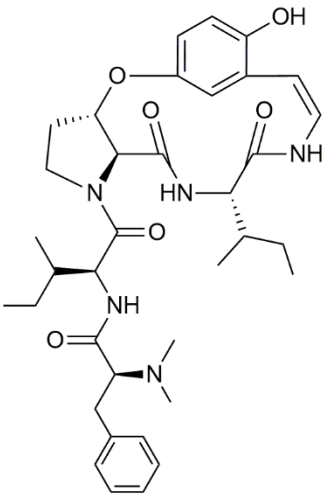
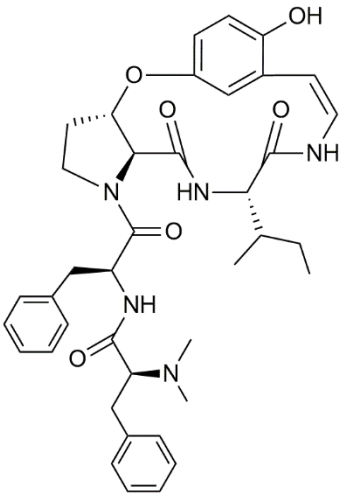


Nummularine-U



Spinanine-B



Spinanine-C

Isolation and structure elucidation of cyclopeptide alkaloids from *Ziziphus nummularia* and *Ziziphus spina-christi* by HPLC-DAD-MS and HPLC-PDA-(HRMS)-SPE-NMR

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ABSTRACT

Seven cyclopeptide alkaloids were isolated from the stem bark of *Ziziphus nummularia* and *Ziziphus spina-christi*. Three previously undescribed compounds were identified: nummularine-U, spinanine-B and spinanine-C, together with the known compounds mauritine-F, nummularine-D, nummularine-E and amphibine-D. For their purification either semi-preparative HPLC with DAD and ESIMS detection or HPLC-PDA-(HRMS)-SPE-NMR was applied, together with conventional separation methods. Their structures were elucidated by spectroscopic means.

Keywords

Ziziphus nummularia, *Ziziphus spina-christi*, Rhamnaceae, cyclopeptide alkaloids

Highlights

- Extraction and fractionation of *Ziziphus nummularia* and *Ziziphus spina-christi* were performed.
- Purification was carried out by HPLC-PDA-(HRMS)-SPE-NMR or semi-preparative HPLC-DAD-MS.
- Three previously undescribed and four known cyclopeptide alkaloids were reported.

1. Introduction

Cyclopeptide alkaloids are polyamide compounds, consisting of a 13-, 14- or 15 membered ring and a side chain, which has either a basic or a neutral character, depending on the presence of a terminal nitrogen atom. The macrocycle is typically composed of a styrylamine unit, a common amino acid and a β-hydroxy-amino acid, whereas the side chain consists of one or two more moieties. Based on these characteristics, the cyclopeptide alkaloids are classified in the 4(13), 4(14), 5(13) or 5(14) subclasses, and compounds from each subclass can be further characterized and classified by their β-hydroxy-amino acid. Their occurrence has been reported in a wide range of families, including the Asteraceae, Celastraceae, Euphorbiaceae, Menispermaceae, Pandaceae, Rubiaceae, Sterculiaceae and Urticaceae, but they are most widely distributed in the Rhamnaceae, and more specifically in the genus *Ziziphus* (Gournelis et al., 1997; Inayat-Ur-Rahman et al., 2001; El-Seedi et al., 2007).

Ziziphus nummularia (Burm.f.) Wight & Arn. (Rhamnaceae) is a thorny shrub that grows in India and Pakistan. It is widely used in ethnomedicine, for example the fruit and root are used to treat diarrhea, and the leaves as an antipyretic and against pain and inflammation. Phytochemical analysis has revealed the presence of flavanoids, phenolic acids, tannins, sterols, saponins, pectin, glycosides and triterpenoic acids. (Goyal et al., 2013; Ray et al., 2015; Rauf et al., 2016). In addition it is a rich source of cyclopeptide alkaloids, with almost 30 different compounds reported until now (Tschesche et al., 1975; Dwivedi et al., 1987; Singh et al., 1995).

Ziziphus spina-christi (L.) Desf. (Rhamnaceae) is a shrub or tree, growing in areas with a sub-tropical climate, for example Egypt, Saudi Arabia, Iraq, Iran and Pakistan. It is used for various medicinal purposes: fruits of this species are used in cases of dysentery and to treat bronchitis and cough, and a decoction of the bark and fresh fruit is used to promote the healing of fresh wounds. It is also used in some inflammatory conditions and against pain.

Previous studies indicated that *Z. spina-christi* contained flavonoids, tannins, sterols, saponins, and triterpenoids (Shahat et al., 2001; Farmani et al., 2016). In addition, eleven different cyclopeptide alkaloids were reported from its stem bark and/or root bark (Tschesche et al., 1974; Shah et al., 1986; Abdel-galil et al., 1991).

Most phytochemical research, and in particular the isolation and structure elucidation of cyclopeptide alkaloids from these two *Ziziphus* species was performed decades ago. Nowadays more sophisticated and more sensitive techniques, such as HPLC-PDA-(HRMS)-SPE-NMR (Wubshet et al., 2015), are available, allowing the identification of minor compounds in plant extracts. Continuing our research program on cyclopeptide alkaloids (Tuenter et al., 2016), it was decided to investigate *Z. nummularia* and *Z. spina-christi* for the presence of yet unknown compounds.

2. Results and Discussion

The bark of *Z. nummularia* and *Z. spina-christi* was extracted with 80% methanol and the crude extracts were fractionated by liquid-liquid partitioning, followed by flash chromatography. The purification of single compounds was performed with either HPLC-PDA-HRMS-SPE-NMR or semi-preparative HPLC with DAD and ESIMS detection. Seven cyclopeptide alkaloids were obtained (**1**-**7**), two from *Z. nummularia* (**1**, **2**) and five from *Z. spina-christi* (**3**-**7**). Their structures (Fig. 1) were elucidated by 1D and 2D NMR experiments and comparison with literature data, and confirmed by (HR)ESIMS.

The NMR data of compound **1** (Table 1 and 2) showed great similarity with the NMR data previously reported for ramosine-A (Lin et al., 2003). Ramosine-A contains a *para*-cyclophane. In the ^{1}H spectrum of **1**, four aromatic proton signals, of which two doublets of doublets, with *J*-values typical for an *o,m*-coupling pattern, and two overlapping signals (also

appearing as doublets of doublets with *J*-values typical for an *o,m*-coupling pattern) were present. This was in agreement with the *para*-cyclophane (14-membered ring type) and not the 13-membered ring type (*meta*-cyclophane). In cyclopeptide alkaloids the chemical shift value of H-9, the β-H of the β-hydroxy-amino acid moiety, is very characteristic, and can be found between 5.00 and 5.50 ppm in a vast majority of compounds. In this case, a multiplet was present at 5.37 ppm. In the COSY spectrum three cross peaks were observed for this proton signal, more specifically with H-8 (δ_H 4.20 ppm, d), H-21A (δ_H 2.22 ppm, m) and H-21B (δ_H 2.58 ppm, m). The proton signals of the CH_2-group in position 21 also showed cross peaks to H-22A (δ_H 3.58 ppm) and H-22B (δ_H 4.06 ppm). H-22A/H-22B did not show other correlations in the COSY spectrum. These data were in agreement with previously reported data for β-hydroxyproline in cyclopeptide alkaloids (more specifically in ramosine-A). The presence of two more amino acids, both isoleucine moieties, could be established based on the NMR data. Based on the HMBC spectrum, the ring bound amino acid could be distinguished from the isoleucine in the side chain, since the *N*-methyl proton signal at 2.62 ppm (H-31) showed a cross peak to the α-carbon of the latter (C-25, δ_C 64.8 ppm). In contrast to ramosine-A, however, the signal that could be assigned to the *N*-methyl protons (δ_H 2.62 ppm, s) integrated for only three protons, whereas in ramosine-A a dimethylated terminal nitrogen atom is present. Moreover, HRESIMS showed a pseudomolecular ion at *m/z* 471.2997, matching with a molecular formula of $C_{26}H_{39}N_4O_4$ $[M+H]^+$, while the molecular formula of ramosine-A is $C_{27}H_{40}N_4O_4$. Therefore it could be concluded that the nitrogen atom of the terminal amino acid moiety of compound **1** contained only a single methyl group, and this compound was named nummularine-U (*N*-desmethylramosine-A), being reported here for the first time.

The spectroscopic data of compound **2** were in agreement with the assignments previously reported for mauritine-F (Cristau et al., 2005).

Compound **1** belongs to the 4(14) subclass of cyclopeptide alkaloids, while compound **2** is of the 5(14) subclass. Both contain proline as the ring bound β-hydroxy-amino acid, and therefore compound **1** can be classified as an amphibine-F type, and compound **2** as an amphibine-B type (Inayat-Ur-Rahman et al., 2001).

Compounds **3** and **4** were both purified by HPLC-PDA-SPE-NMR, and their HRESIMS measurements produced pseudomolecular ions at *m/z* 648.3780 ($C_{36}H_{50}O_6N_5$, $[M+H]^+$) and *m/z* 682.3555 ($C_{39}H_{48}O_6N_5$, $[M+H]^+$), respectively. Their NMR spectra showed the typical pattern of cyclopeptide alkaloids, and allowed to identify some amino acid moieties, that were reported before for paliurine-C and jubanine-A (Tschesche et al., 1976; Lin et al., 2000).

These compounds both contain a 13-membered ring, consisting of a 2-methoxystyrylamine, an isoleucine and a β-hydroxyproline moiety, but differ in their side chain. Paliurine-C contains an additional isoleucine and a terminal *N*-dimethylated phenylalanine amino acid moiety, whereas jubanine-A has two phenylalanine moieties, the terminal one being *N*-dimethylated.

For the structure elucidation of compound **3**, again H-9 could be used as the starting point. This proton had a chemical shift of 5.27 ppm and showed cross peaks to δ_H 2.28 and 2.59 ppm (H-21A and H-21B, respectively) and to δ_H 4.37 (H-8). As in compounds **1** and **2**, the β-hydroxy-amino acid was identified as a β-hydroxyproline, as deduced from the COSY and HMBC data. As for the terminal amino acid moiety, the protons of the *N*-dimethyl group showed a cross peak in the HMBC spectrum to C-32 at 71.5 ppm. H-32 (δ_H 3.31 ppm) on its turn was connected to a CH_2-group (δ_H 2.94 ppm and 3.05 ppm, H-33A and H-33B), as deduced from the COSY spectrum. The HMBC spectrum showed correlations of these protons with δ_C 139.0 and 130.2 ppm, signals typical for an aromatic moiety. Indeed, further inspection of the COSY and HMBC spectrum led to the identification of a benzyl structure, and the terminal amino acid was identified as *N*,*N*-dimethylphenylalanine. Based on the 2D

NMR spectra of compound **3**, two more amino acid moieties could be identified, being both isoleucine moieties. Due to overlap of the signals of C-7 and C-31 in the HMBC spectrum, it was not possible to determine which NMR signals could be assigned to the ring bound isoleucine moiety and which to the isoleucine amino acid in the side chain. However, comparison with the ^{13}C chemical shifts previously reported for paliurine-C (Lin et al., 2000), allowed to discriminate both sets of signals. Especially the chemical shift value of the α-carbon of the amino acid is characteristic, since the α-carbon of the ring-bound isoleucine moiety was reported to have a more downfield shift (δ_C 60.2 ppm), compared to the α-carbon of the amino acid in the side chain (δ_C 53.8 ppm). Lin et al. also investigated the configuration of the α-carbon atoms in the intermediate and terminal amino acid of paliurines-A, -B, -C, -D and -F, by comparison with ^{13}C NMR data of comparable dipeptides. The chemical shift values that were observed in our study for compound **3** were closest to the values reported for the LL form of the dipeptide, indicating the same configuration for both amino acid moieties in the side chain.

For compound **4**, the β-hydroxy-amino acid was identified as β-hydroxyproline and the terminal amino acid as *N*,*N*-dimethylphenylalanine, in the same way as described for compound **3**. The ^{1}H spectrum showed the characteristic pattern for a 1,2,4-trisubstituted benzene (δ_H 6.76, dd, 8.9 and 2.9 Hz, H-12; δ_H 6.84, d, 8.9 Hz, H-13; δ_H 6.71, d, 2.9 Hz, H-16), typically found for cyclopeptide alkaloids with a 13-membered ring (*meta*-cyclophane), substituted in position 14 (for compound **3**, the same moiety was identified, based on the 2D data). HMBC cross peaks of both H-2 and H-5 with C-4, allowed to determine the α-proton of the ring bound amino acid, and further inspection of the 2D spectra led to the identification of this as an isoleucine moiety. One other amino acid could be identified as a phenylalanine moiety, which could only be positioned as the intermediate amino acid in the side chain.

Thus, according to the NMR data, the structures of compound **3** and **4** matched to a great extent with those of paliurine-C and jubanine-A, respectively. However, in the ^{1}H-NMR and HSQC spectra of compounds **3** and **4**, no signal corresponding to the methoxy group could be observed. This led to the conclusion that the two compounds that were purified here, possessed a 2-hydroxystyrylamine instead of a 2-methoxystyrylamine moiety. This hypothesis was in agreement with the obtained HRESIMS data. Taken all data in consideration, compounds **3** and **4**, which to the best of our knowledge have not been reported before, are named spinanine-B (*O*-desmethylpaliurine-C) and spinanine-C (*O*-desmethyljubanine-A).

Furthermore, three other compounds were identified in *Z. spina-christi*: nummularine-D (**5**), nummularine-E (**6**) and amphibine-D (**7**), by comparison with reported spectral data (Tschesche et al., 1972; Tschesche et al., 1975). These compounds were reported before from other sources, but this is the first time they have been identified in *Z. spina-christi*.

The absolute configuration of nummularine-D and –E has not been reported before. However, from the NMR data of the ring bound β-hydroxy-amino acid, β-hydroxyphenylalanine, the configuration of this moiety could be deduced. The C-8/C-9 chemical shifts were 56.8/80.9 ppm and 56.7/81.2 ppm, respectively, for compounds **5** and **6**, and the *J* values of H-9 were close to 8 Hz. These data indicated that the β-hydroxyphenylalanine moiety is present in the L-*erythro* form (Gournelis et al., 1997). The configuration of the two amino acid moieties in the side chain of amphibine-D (**7**) could be deduced by comparing the ^{13}C chemical shift values to those reported by (Lin et al., 2000) for the Leu(OMe)-Phe(NMe_2) dipeptide. Our values were consistent with those reported for the LL-dipeptide, which led to the conclusion that in amphibine-D both the isoleucine amino acid moiety and the phenylalanine amino acid moiety are present in their L-configuration. Considering the fact that the vast majority of cyclopeptide alkaloids are composed of L-amino acids, the L-configuration was also adopted for the other amino acids present in cyclopeptide

alkaloids **1**-**7** reported here, although this could not be confirmed by experimental evidence for all chiral centers (Fig. 1).

In the present work two compounds of the integerrine type were identified in *Z. spina-christi*: nummularine-D (**5**) and nummularine-E (**6**). The other three cyclopeptide alkaloids, spinanine-B (**3**), spinanine-C (**4**) and amphibine-D (**7**), belong to the amphibine-B type (5(14) cyclopeptide alkaloids with a β-hydroxyproline moiety).

3. Conclusion

The phytochemical investigation of the stem bark of *Z. nummularia* and *Z. spina-christi* with advanced spectroscopic techniques led to the isolation of seven cyclopeptide alkaloids. Three compounds were reported for the first time, i.e. nummularine-U (**1**) in *Z. nummularia*, and spinanine-B (**3**) and spinanine-C (**4**) in *Z. spina-christi*. In addition, nummularine-D (**5**), nummularine-E (**6**) and amphibine-D (**7**) were reported for the first time in *Z. spina-christi*.

4. Experimental

4.1 General experimental procedures

Dichloromethane, chloroform, ethyl acetate, methanol, acetonitrile (HPLC-quality), hydrochloric acid (37%), sulfuric acid and ammonia (25%) were purchased from Fisher Chemical (Loughborough, UK). Formic acid, glacial acetic acid, trichloroacetic acid, potassium iodide, hydrogen hexachloroplatinate IV hydrate and *n*-butanol were supplied by Acros Organics (Geel, Belgium). Methanol-d_4 (99.8% D) was from Sigma-Aldrich (Steinheim, Germany). Bismuthsubnitrate was purchased from Merck (Darmstadt, Germany) and cerium sulphate was purchased from Roth (Karlsruhe, Germany). Milli-Q water was prepared with a water purification system of Merck Millipore (Bedford, MA, USA).

A Grace Reveleris X2 flash chromatography system (Lokeren, Belgium) was used for fractionation of the plant material. TLC was performed on NP F_{254} plates (20 cm × 20 cm) from Merck (Darmstadt, Germany) and the spots were observed under UV light (254 and 366 nm) and under visible light after spraying with Dragendorff reagent, iodoplatinate reagent and cerium sulphate reagent (Wagner et al., 1996).

A semi-preparative HPLC system with DAD and ESIMS detectors was used for isolation of pure compounds and was comprised of a sample manager, injector and collector (2767), a quaternary gradient module (2545), a System Fluidics Organizer, an HPLC-pump (515), a photodiode array detector (2998) and a Micromass Quattro mass spectrometer with TQD, all supplied by Waters (Milford, MA, USA). MassLynx version 4.1 was used to process the data.

Two different HPLC-SPE-NMR configurations were used, one HPLC-PDA-SPE-NMR system, consisting of an Agilent 1200 series HPLC system with an in-line solvent degasser, quaternary pump, autosampler, column compartment, and a diode-array detector. The other an HPLC-HRMS-PDA-SPE-NMR system, consisting of an Agilent 1260 series HPLC system with built-in degasser, quaternary pump, autosampler, thermostatted column compartment and diode-array detector, where a flow splitter directed 1% of the eluate to a micrOTOF-Q II mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an electrospray ionization (ESI) interface. In both configurations, the eluate was diluted with water by means of a K-120 HPLC pump (Knauer, Berlin, Germany). Samples were collected using a Prospekt-2 SPE-unit (Spark Holland, Emmen, The Netherlands) using 2 mm GP Resin cartridges, and prepared for NMR using a Gilson Liquid Handler 215 (Gilson, Middleton, WI, USA). The systems were controlled with Hystar version 3.2 software (Bruker Daltonik) and Prep Gilson ST Version 1.2 (Bruker Biospin).

Optical rotations were determined on a JASCO P-2000 spectropolarimeter (Easton, MD, USA) with Spectramanager software and with methanol as blank.

NMR spectra were recorded on two different instruments. The first one was a Bruker Avance III system (Rheinstetten, Germany), operating at 600.13 MHz for ^{1}H and at 150.89 MHz for ^{13}C NMR spectra, equipped with a Bruker SampleJet autosampler and a cryogenically cooled inverse triple-resonance 1.7 mm TCI probe (Bruker Biospin). Standard Bruker pulse sequences were used. Icon-NMR (version 4.2, Bruker Biospin) was used for controlling automated acquisition of NMR data (temperature equilibration to 300 K, optimization of lock parameters, gradient shimming, and setting of receiver gain). NMR data processing was performed with Topspin (version 3.1, Bruker Biospin). The second spectrometer was a Bruker DRX-400 equipped with either a 3 mm inverse broadband (BBI) probe or a 5 mm dual ^{1}H/^{13}C probe using standard Bruker pulse sequences and operating at 400.13 MHz for ^{1}H and at 100.62 MHz for ^{13}C NMR spectra. The spectra were processed with Topspin version 1.3. All NMR spectra were recorded in methanol-d_4.

An Agilent QTOF 6530 mass spectrometer (Santa Clara, CA, USA) with MassHunter version B.06 software was used to perform accurate mass measurements. The mass spectrometer was operated in ESI+ mode at a resolution of 20,000. Calibration was done externally, and the samples were measured after direct infusion.

4.2 Plant materials

Stem bark of *Ziziphus nummularia* (Burm.f.) Wight & Arn. (Rhamnaceae) and *Ziziphus spina-christi* (L.) Desf. (Rhamnaceae) was collected in July and August 2012 in Abdul Khail and in Giloti, Pakistan, respectively. The identification was done by Dr. Mushtaq Ahmad and voucher specimens (ABD/NZ & Khar khar / Khani and X3/X1 respectively) were deposited at the Herbarium of Pakistan, Quaid-i-Azam University, Islamabad, Pakistan.

4.3 Extraction and isolation

The plant material of both plants was dried and milled before extraction. 1.5 kg of stem bark of *Z. nummularia* was percolated with 64 L of 80% methanol. After evaporation of the solvent under reduced pressure and freeze-drying, 162 g of crude extract was obtained. The crude extract was dissolved in 50% methanol/50% water and acidified to pH < 3 with 2 M HCl. Then, liquid-liquid partition was carried out with dichloromethane. Next, the pH of the acidified phase was increased to > 9 by adding NH_3 (25%), followed by a second liquid-liquid partition with dichloromethane. In this way three fractions were obtained: CH_2Cl_2 (I), CH_2Cl_2 (II) and CH_3OH/H_2O (pH > 9) (III). TLC analysis of these three fractions was performed with a mobile phase of $CH_3OH/CHCl_3$/formic acid (10:90:2). TLC indicated that alkaloids were present in the CH_2Cl_2 (II) phase (0.71 g), and further fractionation of 0.56 g of this fraction was performed by means of flash chromatography. A GraceResolv 40 g silica column was used with a mobile phase consisting of dichloromethane (A) and methanol (B) at a flow rate of 30 mL/min. During the run, the ratio of A:B changed stepwise from 100:0 to 0:75 in a time span of 104 min. ELSD and UV detection at 254 nm and 366 nm were used. Throughout the whole experiment the eluent was collected in test tubes, based on the ELSD and UV absorption intensity. Taking into account the obtained chromatograms and the results of a TLC analysis of the collected eluates, which was performed as described above, test tubes showing a similar pattern were combined. This resulted in 15 fractions. TLC analysis on these fractions with the aforementioned spraying reagents and an HPLC screening to assess the complexity of each fraction was then performed. Taking into account the results of both the TLC analysis and HPLC screening and also the amount of each fraction, fractions ZN7 (57.9 mg) and ZN8 (46.5mg) were selected for further analysis. The isolation of single compounds was then performed by semi-preparative HPLC with DAD and MS detection. More detailed information about the chromatographic conditions and the settings of the DAD and mass spectrometer is provided in the supplementary information.

With regard to *Z. spina-christi*, 2.1 kg of stem bark was treated in a similar way as the stem bark of *Z. nummularia*, using 78.6 L of 80% methanol to prepare the crude extract (238 g). 225 g of this crude extract was then submitted to an acid-base liquid-liquid partitioning, as described above. TLC analysis of the three obtained fractions with $CH_3OH/CHCl_3/NH_3$ (5:95:1) indicated that also in this case the CH_2Cl_2 (II) fraction (1.86 g) contained the highest amount of alkaloids and 1.5 g of this fraction was further separated into 9 subfractions by flash chromatography. A GraceResolv 80 g silica column was used with dichloromethane (A), ethyl acetate (B) and methanol (C) as the solvents and a flow rate of 30 mL/min. During the run, the ratio of A:B changed stepwise from 100:0 to 0:100 in a time span of 25 min, followed by a change from B:C from 0:100 to 50:50 in 40 min. The settings of the detector and handling of the samples were the same as for *Z. nummularia*. TLC analysis with the aforementioned spraying reagents, together with an HPLC screening to assess the complexity of each fraction and the amount of each fraction available led to the selection of fractions ZSC6 (15.3 mg), ZSC7 (97.7 mg) and ZSC8 (105.1 mg) for further analysis.

Fraction ZSC6 was analyzed by HPLC-PDA-HRMS-SPE-NMR. Ten microliter of the sample, with a concentration of 15 mg/mL, was injected repetitively in the HPLC system. Separation was accomplished using a Phenomenex C18(2) Luna column (150 mm × 4.6 mm, 3 μm particle size, 100 Å pore size), which was kept at 40 °C. The mobile phase consisted of 95% water/5% acetonitrile/0.1% formic acid (A) and 5% water/95% acetonitrile/0.1% formic acid (B) with the following gradient: 0 min 100:0 (A:B), 17 min 50:50, 20-25 min 0:100. The flow rate was 0.5 mL/min and the flow set for the K-120 pump was 1 mL/min. DAD spectra were recorded between 190 and 950 nm. Mass spectra were acquired in the positive ion mode in the range of *m/z* 50 to 1000, using a drying temperature of 200 °C, capillary voltage of 4100 V, nebulizer pressure of 2.0 bar and a dry gas flow of 7 L/min. The eluate was trapped

as long as the UV absorption at 228 nm was higher than 600 mAu. Subsequently, the cartridge was eluted into 1.7 mm o.d. NMR tubes (Bruker Biospin, Rheinstetten, Germany) with methanol-d_4 (final volume in tube 35 μL).

Fraction ZSC7 was further separated by HPLC-DAD-SPE-NMR. The injection volume was 20 μL and the sample concentration 8.4 mg/mL. An XBridge column (C18, 4.6 × 250 mm, 5μm, Waters (Milford, MA, USA)) was used with water + 0.1% ammonia (A) and acetonitrile (B) as the mobile phase. The flow rate was 0.8 mL/min (2.4 mL/min for the K-120 pump) and the gradient was: 0 to 5 min 70:30 (A:B), 35 min 50:50, 40 to 45 min 0:100. DAD spectra were recorded between 190 and 450 nm. The threshold for trapping was set at 375 mAu for the UV signal at 210 nm. Trapped compounds were eluted with methanol-d_4 in 3 mm o.d. NMR tubes.

Semi-preparative HPLC-DAD-MS was used for isolation of two compounds from fraction ZSC8. More detailed information regarding this can be found in the supplementary information.

1D (^{1}H, ^{13}C) and 2D (COSY, HSQC, HMBC) NMR spectra were recorded for all compounds, and for compounds which were not described previously, accurate mass measurements were performed. nummularine-U (**1**) was isolated by semi-preparative HPLC-DAD-MS from ZN7 and ZN8 and mauritine-F (**2**) only from fraction ZN8. spinanine-B (**3**) and spinanine-C (**4**) were identified in fraction ZSC7 after HPLC-SPE-NMR. From fraction ZSC6 nummularine-D (**6**) was purified by HPLC-SPE-NMR, whereas nummularine-E (**7**) was obtained by semi-preparative HPLC-DAD-MS from fraction ZSC8, together with amphibine-D (**5**).

4.3.1 *Nummularine-U (**1**)*

White powder (1 mg); UV λ_{max} 207 nm; ^{1}H and ^{13}C NMR spectroscopic data: see Table 1 and 2, respectively; HRESIMS *m/z* 471.2997 $[M+H]^+$, (calcd for $C_{26}H_{39}N_4O_4$, 471.2966).

*4.3.2 Mauritine-F (**2**)*

White powder (6 mg); UV λ_{max} 204 nm; $[\alpha]_D$ -173.3 (*c* 0.6, CH_3OH); ^{1}H and ^{13}C NMR spectroscopic data: see Table 1 and 2, respectively; ESIMS *m/z* 562.3 $[M+H]^+$.

*4.3.3 Spinanine-B (**3**)*

White powder (4 mg); UV λ_{max} … nm; $[\alpha]_D$ -218.3 (c 0.4, CH_3OH); ^{1}H and ^{13}C NMR spectroscopic data: see Table 1 and 2, respectively; HRESIMS *m/z* 648.3780 $[M+H]^+$, (calcd for $C_{36}H_{50}N_5O_6$, 648.3756).

*4.3.4 Spinanine-C (**4**)*

(< 1 mg); UV λ_{max} 202, 268, 322 nm; ^{1}H and ^{13}C NMR spectroscopic data: see Table 1 and 2, respectively; HRESIMS *m/z* 682.3555 $[M+H]^+$, (calcd for $C_{39}H_{48}N_5O_6$, 682.3599).

*4.3.5 Nummularine-D (**5**)*

UV λ_{max} 225 nm; ^{1}H and ^{13}C NMR spectroscopic data: see Table 1 and 2, respectively; ESIMS *m/z* 521.3 $[M+H]^+$.

*4.3.6 Nummularine-E (**6**)*

White powder (27 mg); UV λ_{max} 214 nm; $[\alpha]_D$ -184.0 (c 0.5, CH_3OH/H_2O/formic acid (50:50:0.1)); ^{1}H and ^{13}C NMR spectroscopic data: see Table 1 and 2, respectively; ESIMS *m/z* 523.3 $[M+H]^+$.

4.3.7 Amphibine-D (7)

White powder (2 mg); UV λ_{max} 202 nm; $[\alpha]_D$ -136.7 (c 0.2, CH_3OH); ^1H and ^{13}C NMR spectroscopic data: see Table 1 and 2, respectively; ESIMS *m/z* 632.4 $[M+H]^+$.

Acknowledgements

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Figures and legends

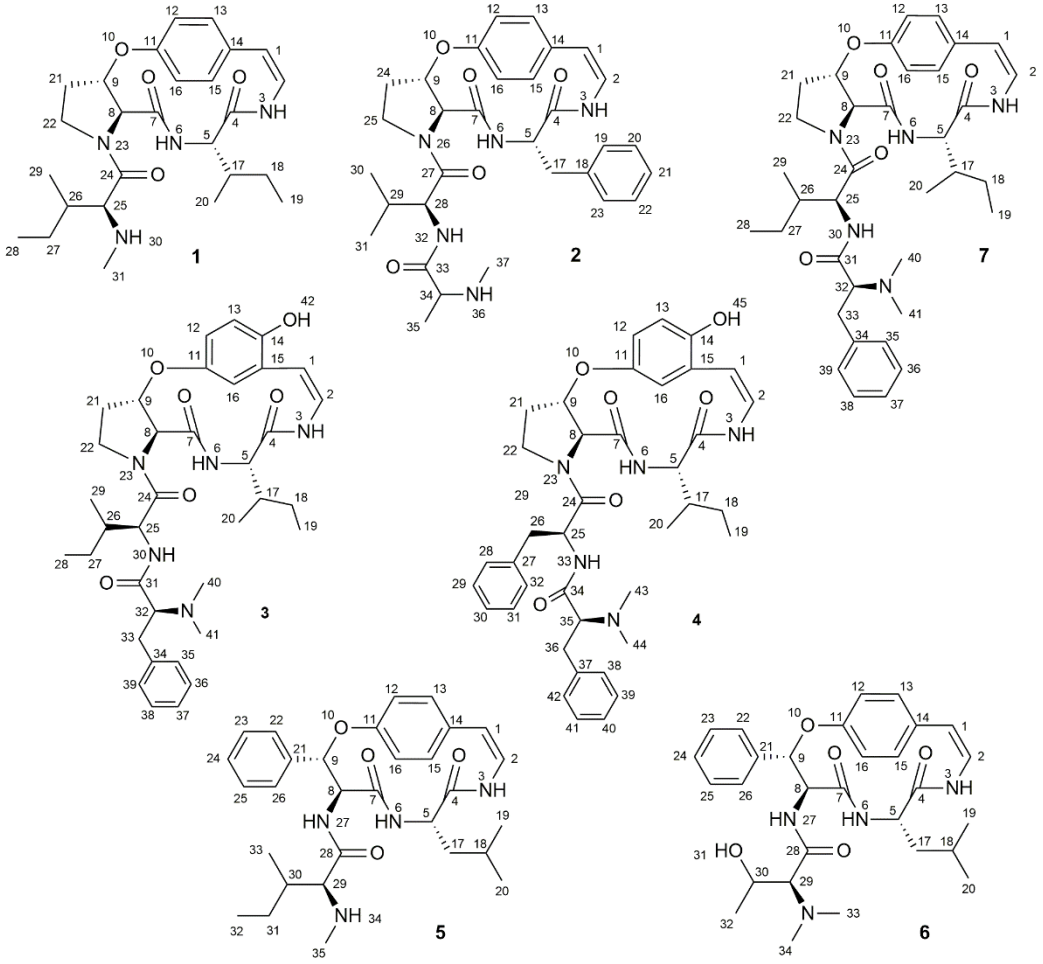

Fig. 1. Chemical structures of compounds **1**-**7**.

Tables

Table 1

^{1}H NMR spectroscopic data (δ_H in ppm, multiplicity (*J* in Hz)) for compounds **1**-**7**.

Position	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	6.77, d (7.5)	6.70, d (7.5)	6.06, d (9.0)	6.05, d (8.9)	6.76, d (7.5)	6.78, d (7.5)	6.75, d (7.5)
2	6.19, d (7.5)	6.11, d (7.5)	6.80, d (9.0)	6.80, d (9.0)	6.25, d (7.5)	6.23, d (7.5)	6.24, d (7.5)
3 (N-H)	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
5	3.91, d (8.0)	4.32, dd (8.3, 5.2)	4.15, d (6.7)	4.20, d (6.6)	4.18, m	4.19, dd (9.2, 5.0)	3.87, d (8.1)
6 (N-H)	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
8	4.20, d (6.6)	4.08, d (6.7)	4.37, d (3.3)	4.34, d (3.0)	4.83[a]	4.87[s]	3.95, d (6.5)
9	5.37, m	5.30, m	5.27, m	5.18, m	5.94, d (8.2)	5.95, d (8.0)	5.35, m
12	7.16, dd (8.5, 2.2)	7.16[a]	6.81[a]	6.76, dd (8.9, 2.9)	7.20, dd (8.4, 2.5)	7.18, dd (8.2, 2.5)	7.17, dd (8.4, 2.1)
13	7.08[a]	7.02[a]	6.86, d (8.9)	6.84, d (8.9)	7.10, d (8.4)	7.09, d (8.2)	7.08, dd (8.4, 2.1)
14	-	-	-	-	-	-	-
15	7.11[a]	7.03[a]	-	-	7.05, dd (8.5, 1.8)	7.05, dd (8.4, 1.8)	7.14[a]
16	7.03, dd (8.2, 2.2)	7.03[a]	6.71, d (2.7)	6.71, d (2.9)	7.24, dd (8.5, 2.5)	7.23, dd (8.4, 2.5)	7.12[a]
17A	1.62, m	2.82, m	1.91, m	1.92, m	1.30, m	1.28*	1.62, m

17B	-	-	-	-	1.41, m	1.40[a]	-
18A	1.07, m	-	1.24, m	1.26, m	1.41, m	1.40[a]	0.99, m
18B	1.47, m	-	1.47, m	1.52, m	-	-	1.40, m
19	0.79, t (7.5)	7.17[a]	0.87, t (7.4)	0.92, t (7.4)	0.84, d (6.3)	0.81, d (5.4)	0.77[a]
20	0.81, d (6.8)	7.23[a]	0.96, d (6.9)	1.00, d (6.9)	0.80, d (6.3)	0.82, d (5.4)	0.78[a]
21A	2.22, m	7.18[a]	2.28, m	2.18, m	-	-	2.14, m
21B	2.58, m	-	2.59, m	2.43, m	-	-	2.56, m
22A	3.58, m	7.23[a]	3.60, m	3.21, m	7.57, d (7.5)	7.58, d (7.5)	3.52, m
22B	4.06, m	-	4.26, m	3.95, m	-	-	4.17, dd (10.4, 9.0)
23	-	7.17[a]	-	-	7.38, t (7.5)	7.40, t (7.5)	-
24A	-	2.21, m	-	-	7.31, t (7.5)	7.33, t (7.5)	-
24B	-	2.58, m	-	-	-	-	-
25A	4.10, m	3.62, m	4.48, d (8.9)	4.87, t (7.4)	7.38, t (7.5)	7.40, t (7.5)	4.39, d (9.0)
25B	-	4.25, t (9.4)	-	-	-	-	-
26A	1.89, m	-	1.74, m	2.84, dd (13.6, 7.1)	7.57, d (7.5)	7.58, d (7.5)	1.7, m
26B	-	-	-	2.99, dd (13.6, 7.1)	-	-	-
27A	1.15, m	-	1.15, m	-	N-H, n.o.	N-H, n.o.	1.10, m

27B	1.54, m	-	1.53, m	-	-	-	1.49, m
28	0.94, t (7.2)	4.47, d (7.5)	0.87, t (7.4)	7.10-7.30[b]	-	-	0.85, t (7.5)
29	1.02, d (6.8)	2.05, m	0.80, d (6.8)	7.10-7.30[b]	2.49, d (5.4)	2.47, d (9.0)	0.76[a]
30	N-H, n.o.	0.93, d (6.6)	N-H, n.o.	7.10-7.30[b]	1.34[a]	3.72, m	N-H, n.o.
31A	2.63, s	0.96, d (6.6)	-	7.10-7.30[b]	0.82[a]	O-H, n.o.	-
31B	-	-	-	-	1.09, m	-	-
32		N-H, n.o.	3.31[s]	7.10-7.30[b]	0.75, t (7.3)	0.91, d (6.2)	3.65, m
33A		-	2.94, dd (13.0, 4.8)	N-H, n.o.	0.64, d (6.9)	1.75, s	3.09, m
33B		-	3.05, dd (13.0, 9.8)	-	-	-	-
34		3.86[a]	-	-	N-H, n.o.	1.75, s	-
35		1.49, d (5.0)	7.19, d (7.5)	3.25, m	1.72, s		7.23, dd (7.4, 2.3)
36A		N-H, n.o.	7.26, t (7.5)	2.90, dd (13.2, 5.1)			7.35[a]
36B		-	-	2.98, dd (13.2, 5.1)			-
37		2.65, s	7.12, t (7.5)	-			7.34[a]
38			7.26, t	7.10-7.30[b]			7.35[a]

		(7.5)		
39		7.19, d	7.10-7.30[b]	7.23, dd
		(7.5)		(7.4, 2.3)
40		2.40, s	7.10-7.30[b]	2.63, s
41		2.40, s	7.10-7.30[b]	2.63, s
42			7.10-7.30[b]	
43			2.25, s	
44			2.25, s	

n.o. = not observed. [s]Overlapping with solvent or water signal. [a]Overlapping signal. [b]Signals of aromatic protons could not be assigned to a specific position, due to overlapping of signals. All NMR spectra were recorded in methanol-d_4.

Table 2

^{13}C NMR spectroscopic data (δ_C in ppm) for compounds **1**-**7**.

Position	1	2	3	4	5	6	7
1	130.4	129.6	110.9	110.3	128.2	128.6	129.8
2	126.3	126.4	121.5	121.2	125.5	125.4	126.6
4	171.6	171.0	170.6	170.2	n.o.	n.o.	171.9
5	59.3	56.1	61.3	61.1	51.4	51.4	59.7
7	172.4	172.1	173.1*	172.4	n.o.	169.6	172.8
8	66.6	66.7	66.2	66.5	56.8	56.7	66.5
9	83.2	83.4	79.0	78.6	80.9	81.2	84.2
11	158.4	158.4	151.8	151.5	n.o.	155.2	158.7
12	118.8	119.6	119.1	118.7	121.2	121.2	119.9
13	131.7	131.9	119.4	118.9	129.3	129.2	132.2
14	133.4	133.0	150.6	150.4	n.o.	131.7	133.6
15	130.6	130.5	123.2	122.9	130.2	130.1	130.9
16	121.7	122.1	112.0	111.8	118.4	118.6	122.3
17	38.5	38.9	36.6	36.7	41.0	41.0	38.5
18	25.2	137.9	26.1	26.1	24.2	24.2	25.3
19	11.0	130.1	11.2 or 10.8*	11.4	21.9	20.9	15.3
20	15.3	129.1	16.1	15.9	20.9	21.9	15.6
21	32.4	127.4	33.7	33.2	n.o.	138.1	32.7
22	47.0	129.1	47.6	47.0	128.1	130.0	47.2

23	-	130.1	-	-	128.0	129.7	-
24	167.8	32.4	171.8	170.9	128.1	129.7	170.7 or 171.5*
25	64.8	46.8	55.2	52.6	128.0	129.7	55.2
26	38.0	-	38.2	38.9	128.1	130.0	38.3
27	25.1	171.8	25.9	137.5	-	-	25.9
28	11.6	57.2	11.2 or 10.8*	127.1-130.1[a]	n.o.	171.0	10.8
29	14.6	31.3	15.3	127.1-130.1[a]	68.6	64.6	11.4
30	-	19.2	-	127.1-130.1[a]	37.6	72.8	-
31	33.1	18.4	172.8*	127.1-130.1[a]	24.3		170.7 or 171.5*
32		-	71.5	127.1-130.1[a]	10.6	20.5	71.0
33		170.5	37.0	-	14.3	40.7	36.4
34		58.1	139.0	172.4	-	40.7	137.7
35		16.3	130.2	71.3	33.7		130.4
36		-	129.5	36.3			129.7
37		31.7	127.5	139.3			128.1
38			129.5	127.1-130.1[a]			129.7
39			130.2	127.1-130.1[a]			130.4

40		42.6	127.1-130.1[a]	42.6
41		42.6	127.1-130.1[a]	42.6
42			127.1-130.1[a]	
43			42.4	
44			42.4	

*Interchangeable. [a]Signals of aromatic carbon atoms could not be assigned to a specific position, overlapping signals. All NMR spectra were recorded in methanol-d_4.

References

Abdel-galil, F. M. and El-Jissry, M. A., 1991. Cyclopeptide alkaloids from *Zizyphus spina-christi*. Phytochemistry 30(4), 1348-1349.

Cristau, P., Temal-Laib, T., Bois-Choussy, M., Martin, M. T., Vors, J. P. and Zhu, J. P., 2005. Total synthesis of Mauritines A, B, C, and F: Cyclopeptide alkaloids with a 1-4-membered paracyclophane unit. Chem. Eur. J. 11(9), 2668-2679.

Dwivedi, S. P. D., Pandey, V. B., Shah, A. H. and Eckhardt, G., 1987. Cyclopeptide Alkaloids from *Zizyphus nummularia*. J. Nat. Prod. 50(2), 235-237.

El-Seedi, H., Zahra, M. H., Goransson, U. and Verpoorte, R., 2007. Cyclopeptide alkaloids. Phytochem. Rev. 6, 143-165.

Farmani, F., Moein, M., Amanzadeh, A., Kandelous, H. M., Ehsanour, Z. and Salimi, M., 2016. Antiproliferative evaluation and apoptosis induction in MCF-7 cells by *Ziziphus spina christi* leaf extracts. Asian Pac. J. Cancer P. 17(1), 315-321.

Gournelis, D. C., Laskaris, G. G. and Verpoorte, R., 1997. Cyclopeptide alkaloids. Nat. Prod. Rep. 14(1), 75-82.

Goyal, M., Ghosh, M., Nagori, B. P. and Sasmal, D., 2013. Analgesic and anti-inflammatory studies of cyclopeptide alkaloid fraction of leaves of *Ziziyphus nummularia*. Saudi J. Biol. Sci. 20(4), 365-371.

Inayat-Ur-Rahman, Khan, M. A., Khan, G. A., Khan, L. and Ahmad, V. U., 2001. Cyclopeptide alkaloids of *Zizyphus* species. J. Chem. Soc. Pakistan 23(4), 268-277.

Lin, H. Y., Chen, C. H., Liu, K. and Lee, S. S., 2003. 14-membered cyclopeptides from *Paliurus ramosissimus* and *P. hemsleyanus*. Helv. Chim. Acta 86(1), 127-138.

Lin, H. Y., Chen, C. H., You, B. J., Liu, K. C. and Lee, S. S., 2000. Cyclopeptide alkaloids from *Paliurus ramossisimus*. J. Nat. Prod. 63(10), 1338-1343.

Rauf, A., Ali, J., Khan, H., Mubarak, M. S. and Patel, S., 2016. Emerging CAM *Ziziphus nummularia* with in vivo sedative-hypnotic, antipyretic and analgesic attributes. 3 Biotech 6.

Ray, S. D. and Dewanjee, S., 2015. Isolation of a new triterpene derivative and *in vitro* and *in vivo* anticancer activity of ethanolic extract from root bark of *Zizyphus nummularia* Aubrev. Nat. Prod. Res. 29(16), 1529-1536.

Shah, A. H., Ageel, A. M., Tariq, M., Mossa, J. S. and Al-Yahya, M. A., 1986. Chemical constituents of the stem bark of *Zizyphus spina-christi*. Fitoterapia 58(6), 452-454.

Shahat, A. A., Pieters, L., Apers, S., Nazeif, N. M., Abdel-Azim, N. S., Vanden Berghe, D. and Vlietinck, A. J., 2001. Chemical and biological investigations on *Zizyphus spina-christi* L. Phytother. Res. 15(7), 593-597.

Singh, B. and Pandey, V. B., 1995. An N-formyl cyclopeptide alkaloid from *Zizyphus nummularia* bark. Phytochemistry 38(1), 271-273.

Tschesche, R., Elgamal, M., Miana, G. A. and Eckhardt, G., 1975. Alkaloids from rhamnaceae—XXVI. Nummularine-D, -E and -F, new cyclopeptide alkaloids from *Ziziphus nummularia*. Tetrahedron 31(23), 2944-2947.

Tschesche, R., Fehlhaber, H. W. and Kaussmann, E. U., 1972. Alkaloids from Rhamnaceae. 13. Amphibine-B, amphibine-C, amphibine-D, amphibine-E, 4 peptide alkaloids from *Ziziphus amphibia* A. Cheval. Chem. Ber.-Recl. 105(9), 3094-3105.

Tschesche, R., Khokhar, I., Spilles, C. and von Radloff, M., 1974. Peptide alkaloids from *Ziziphus spinachristi*. Phytochemistry 13(8), 1633.

Tschesche, R., Khokhar, I., Wilhelm, H. and Eckhardt, G., 1976. Alkaloids from Rhamnaceae.27. Jubanine-A and jubanine-B, new cyclopeptide alkaloids from *Ziziphus jujuba*. Phytochemistry 15(4), 541-542.

Tuenter, E., Exarchou, V., Balde, A., Cos, P., Maes, L., Apers, S. and Pieters, L., 2016. Cyclopeptide Alkaloids from *Hymenocardia acida*. J. Nat. Prod. 79(7), 1746-1751.

Wagner, H. and Bladt, S., 1996. Appendix A: Spray Reagents. In: Plant drug analysis, a thin layer chromatography atlas. 2nd. ed. Springer-Verlag, Berlin - Heidelberg - New York, pp. 359-364.

Wubshet, S. G., Moresco, H. H., Tahtah, Y., Brighente, I. M. G. and Staerk, D., 2015. High-resolution bioactivity profiling combined with HPLC-HRMS-SPE-NMR: alpha-glucosidase inhibitors and acetylated ellagic acid rhamnosides from *Myrcia palustris* DC. (Myrtaceae). Phytochemistry 116, 246-252.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/...