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Selective apoptosis-inducing activity of crinum-type Amaryllidaceae alkaloids

James McNulty [a,*], Jerald J. Nair [a], Carles Codina [b], Jaume Bastida [b], Siyaram Pandey [c], Jenny Gerasimoff [c], Carly Griffin [c]

[a] Department of Chemistry, McMaster University, 1280 Main Street West, Hamilton, Ont., Canada L8S 4M1
[b] Departament de Productes Naturals, Facultat de Farmacia, Universitat de Barcelona, 08028, Barcelona, Spain
[c] Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ont., Canada N9B 3P4

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Abstract

The selective apoptosis-inducing activity of Amaryllidaceae alkaloids belonging to the crinane-type is reported. A mini-library of natural and synthetic crinane alkaloids was assembled. Biological screening indicated crinamine **4** and haemanthamine **9** to be potent inducers of apoptosis in tumour cells at micromolar concentrations. Structure-activity relationships demonstrated the requirement for both an alpha-C2 bridge and a free hydroxyl at the C-11 position as pharmacophoric requirements for this activity.

Keywords: Amaryllidaceae; Apoptosis-induction; Anticancer agent; Alkaloid

1. Introduction

Alkaloids produced by plants belonging to the Amaryllidaceae family (Cook and Loudon, 1952; Wildman, 1968; Martin, 1987) have attracted the attention of chemists for well over a century due to the challenging isolation and structural problems presented coupled with reports of various biological activities. While the medicinal use of extracts from such plants can be dated to the times of Hippocrates and Pliny (Pettit et al., 1993), the more recently demonstrated potent anticancer activity of certain Lycorane-type alkaloids (see Scheme 1) and the selective, reversible acetylcholinesterase inhibitory activity demonstrated by certain Galanthamine-type alkaloids (Marco-Contelles et al., 2006) has fuelled the clinical advance of amaryllidaceae alkaloids and their synthetic derivatives (Rinner and Hudlicky, 2005) toward the development of anti-tumour and anti-Alzheimer's therapeutics.

Structurally, the Amaryllidaceae alkaloids are related as a consequence of their biogenesis from the common amino-acid derived precursor norbelladine **1** (Scheme 1) from which, through varied skeletal rearrangements, the major Amaryllidaceae structural types are derived. Three classic structural types are recognised (McKillop, 1969) shown in Scheme 1 as those that contain or are derived from a lycorane, crinane or galanthamine core, although several other types and more complex modifications are also now well known (Jin, 2005).

From a biomedical perspective, significant attention has focussed on the Lycorane-type constituents such as pancratistatin **2** (Scheme 1) (Pettit et al., 1993) and its natural and synthetic derivatives (Rinner and Hudlicky, 2005) in view of the potent anticancer activity demonstrated (Scheme 2). Although the mechanism of action of the pancratistatin sub-family is still elusive, work conducted in our laboratories has recently established that these alkaloids are cytostatic through initiation of apoptosis (McLachlan

* Corresponding author. Tel.: +905 525 9140x27393; fax: +905 522 2509.
E-mail address: jmcnult@mcmaster.ca (J. McNulty).

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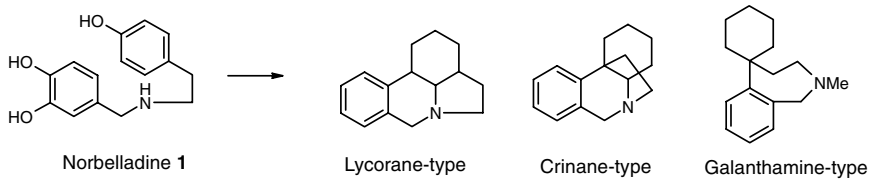
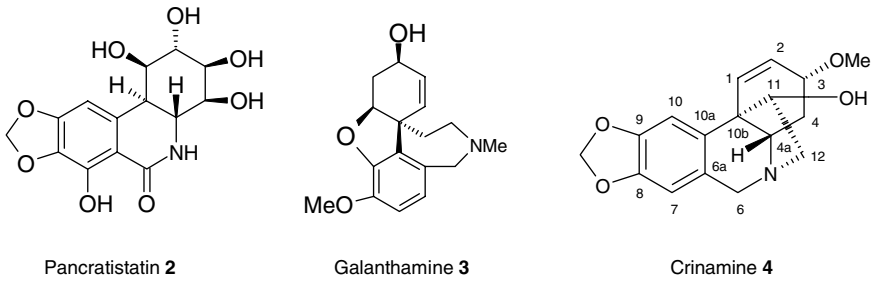


Scheme 1. Biosynthetic origin of the Amaryllidaceae alkaloids.



Scheme 2. Selected biologically active Amaryllidaceae constituents.

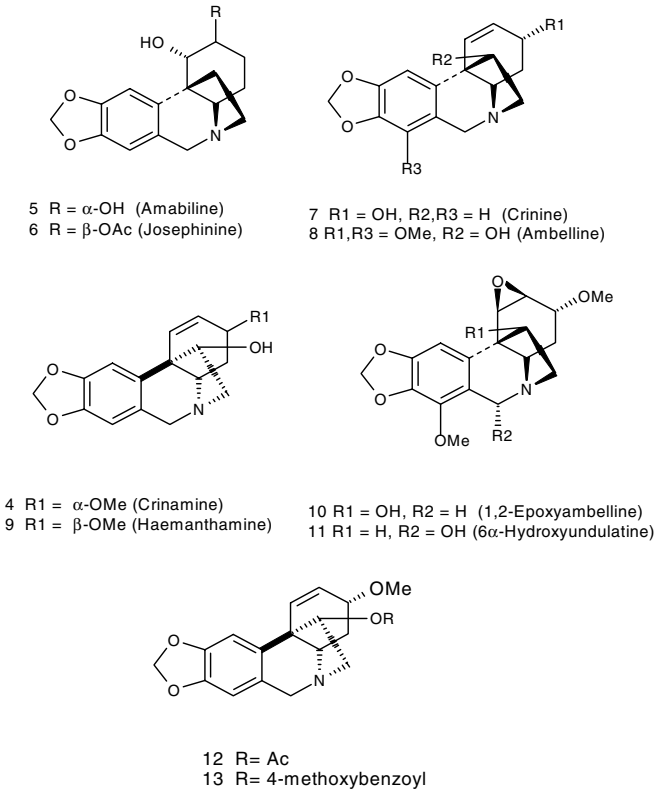
et al., 2005) or programmed cell death, through a pathway that involves the early activation of caspase-3 followed by flipping of phosphatidyl serine (Kekre et al., 2005). Furthermore, these alkaloids have demonstrated selective cytotoxicity towards certain tumour cells providing an encouraging incentive for the development of potentially tumour cell-line targeted chemotherapeutics. On the other hand, significant attention has focussed on Galanthamine-type alkaloids such as galanthamine **3** (Scheme 2) and sanguinine in the treatment of Alzheimer's and other neuro-degenerative diseases where lycorane and crinane-type alkaloids have been shown to be significantly less active (Houghton et al., 2006). Indeed galanthamine hydrobromide, under the generic name Reminyl, is the first Amaryllidaceae alkaloid to be approved as a prescription drug in the pharmaceutical treatment of a human disease. Galanthamine **3** exerts its biological activity through a mechanism involving inhibition of acetylcholinesterase of relevance in the progression of neuro-degenerative diseases.

The significant and quite different biological activities demonstrated by the Lycorane and Galanthamine type alkaloids naturally draws attention to bases of the central crinane-type (Scheme 1). Crinum alkaloids have previously been shown to exhibit a range of biological activities (Tram et al., 2002). The cytotoxicity of crinamine **4** to the malaria parasite and to a series of tumor cell lines was previously described (Likhitwitayawuid et al., 1993), while 6-hydroxycrinamine was shown to be active against mouse melanoma cells (Nair et al., 1998). The discovery of compounds that initiate programmed cell death is significant, in particular when such activity could be related to the pancratistatin class, given the selective cytotoxicity that has been demonstrated by members of this class. The present paper reports on a structure-activity study of eight natural and two synthetic crinane-type Amaryllidaceae alkaloids, and the ability of two of them to initiate apoptosis in a selective manner. Structural requirements, including a free secondary hydroxyl group at C-11 and the alpha-5,10b-ethano bridge, for this apoptosis-inducing activity are revealed for the first time.

2. Results and discussion

The selection of ten crinane-type alkaloids shown in Scheme 3 was assembled and this mini-library was screened for its ability to induce apoptosis selectively in rat liver hepatoma (5123tc) but not in a non-cancerous cell line, human embryonic kidney (HEK-293t) cells.

Fig. 1 is a representative graph displaying the apoptotic index of 5123tc cells after 48 h of treatment with Amaryllidaceae alkaloids **4** through **11**. Cells were treated with 25 μM final concentration of the alkaloids as outlined under the experimental section; the selective apoptosis inducing activity demonstrated by crinamine **4** and haemanthamine **9** is striking. Both had an ability to effectively induce apoptosis at 25 μM after 48 h in 5123tc cancer cells, with 85% to >90% of all cells displaying characteristic apoptotic morphology, condensed nuclei, brightly stained with Hoechst dye. In parallel to Hoechst staining, cells were also stained with Trypan Blue dye to evaluate the extent of necrotic cells. The percentage of cells showing apoptosis was higher than the number of Typan Blue positive cells, indicating that many of the apoptotic cells still have an intact cell membrane (data not shown). Fig. 2 shows the morphological changes visualized by Hoechst staining following treatment of 5123tc cells with alkaloid **4** at increasing concentrations for 72 h. Hoechst is a fluorescent DNA-binding dye that stains the condensed nuclei of apoptotic cells brighter than the intact nuclei of normal



Scheme 3. Crinane-type Amaryllidaceae alkaloids screened in the present study.

cells. The effective dose, that is the concentration of test compound capable of inducing apoptosis in 50% of cells, hereafter referred to as the ED50, was determined to be 12.5 μM for alkaloid **4** and 15 μM for alkaloid **9**. These values were calculated using a dose curve at 72 h treatment as shown in Fig. 3. As a further indication of induction of programmed cell death, annexin-V binding assays were also conducted on the 5123tc cells treated with alkaloids

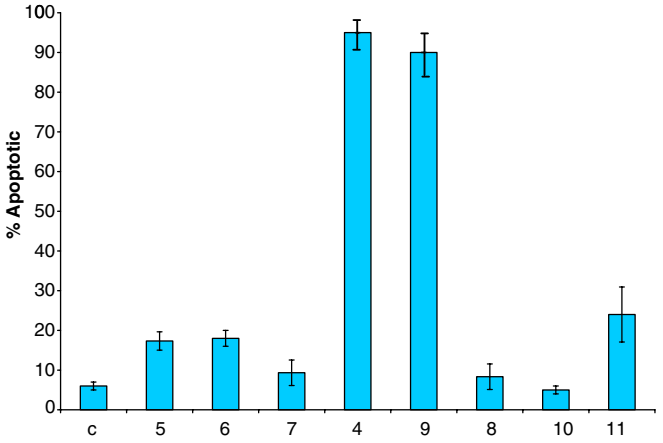


Fig. 1. Apoptotic index in rat hepatoma (5123tc) cells: Amaryllidaceae alkaloids **4** and **9** are highly effective at 25 μM after a 48 h treatment. Cancerous 5123 cells were treated for 48 h with the alkaloids at a concentration of 25 μM; **4** and **9** generated apoptotic indices of ~90%. The standard error was calculated using Microsoft Excel software with data obtained from three separate experiments.

4 and **9**. These studies clearly indicated the flipping of phosphatidyl serine from the inner to outer leaflet of the plasma membrane, a further hallmark of apoptosis as shown in Fig. 4. Although alkaloids **4** and **9** are a little less potent than pancratistatin **2**, the binding assays and morphological changes were otherwise similar to those seen previously with **2** (Kekre et al., 2005).

More significantly, alkaloids **4** and **9** did not induce apoptosis in a non-cancerous human embryonic kidney (HEK 293t) cell-line when treated at similar concentrations as shown in Fig. 5. Taken together, these results indicate that of all the crinane-type alkaloids, **4** and **9** effectively induce apoptosis in rat liver hepatoma (5123tc), while the lack of apoptosis demonstrated in a non-cancerous cell line shows promise in terms of selectivity similar to that demonstrated by pancratistatin and derivatives. The selective

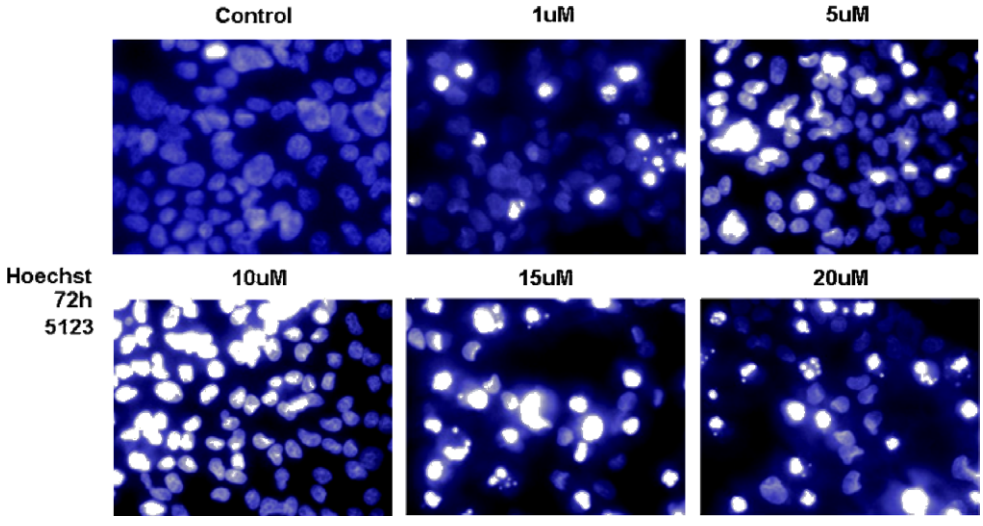


Fig. 2. Hoechst staining to determine the ED50 of alkaloid **4**. 5123tc cells were treated with various concentrations of **4** for 72 h. Apoptotic nuclei appear brighter and more fragmented than healthy nuclei, which are less bright and intact.

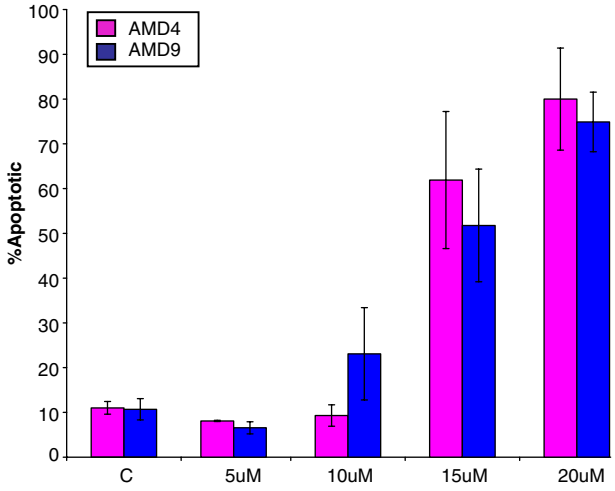


Fig. 3. 5123tc cells were treated with various concentrations of alkaloid **4** and **9** for 72 h. The effective dose was calculated as the concentration inducing apoptosis in 50% of cells.

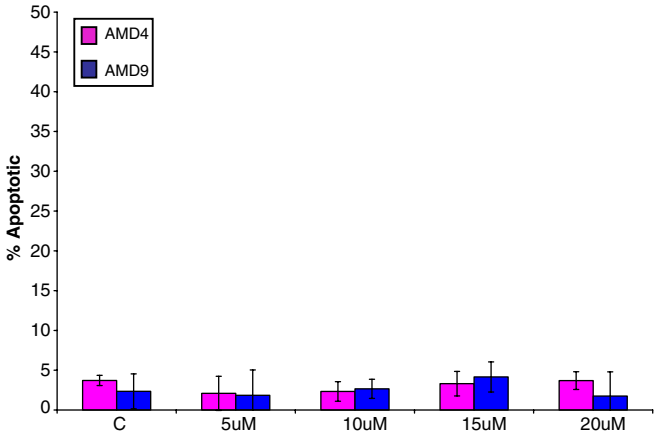


Fig. 5. No induction of apoptosis in normal human embryonic kidney 293t cells by treatment with alkaloids **4** and **9** for 72 h at various concentrations. Cells were grown, treated and stained with Hoechst cell-permeable dye as described. The standard error was calculated using Microsoft Excel software with data obtained from three separate experiments.

apoptosis-inducing activity possessed by alkaloids **4** and **9**, evident from Fig. 1, clearly indicates the requirement of the alpha-5,10b-ethano bridge as an important pharmacophoric element. Biological activities including anti-proliferative action and inhibition of protein synthesis by haemanthamine **9** has also been previously shown (Hohmann et al., 2002; Jimenez et al., 1976). For example, alkaloids **7** and **8** possessing the beta-5,10b-ethano bridge show little activity irrespective of the hydroxyl and methoxy functionality similar to active analogs **4** and **9**. The C1–C2 double bond is present in both of the active alkaloids having the alpha-bridge and also the non-active derivatives containing the beta-bridge. While the double bond in **4** and **9** no doubt contributes a conformational role, the possibility that it is a pro-pharmacophoric element, for example as a precursor to an epoxide or diol, remains a possibility.

It is also clear from the similar apoptosis-inducing activity shown by alkaloids **4** and **9** that the stereochemistry at the C3-methoxy group has little impact and is not likely a pharmacophoric element. On the other hand, it is well known in the pancratistatin (**2**, Scheme 2) series of apoptosis-inducing alkaloids (lycorane type) that the correct positioning of one or more free hydroxyl group greatly influences the anticancer activity observed (McNulty et al., 2005). It was therefore of importance to investigate the role of the free C11-hydroxyl group on the biological activity. Given the quantity of crinamine **4** available, we were able to probe limited structure-activity-relationships (SAR) pertaining to the C-11 hydroxyl group. Derivatives of the most potent apoptosis inducing alkaloid crinamine **4** were prepared through acylation of the free bridging hydroxyl with acetic anhydride and 4-methoxybenzoyl chloride, respectively yielding alkaloids **12** and **13**. These two derivatives were shown to be void of any apoptosis inducing effect. These results highlight the importance of the free hydroxyl group on the alpha-C2 bridge for induction of apoptosis. Again, these results are reminiscent of the requirement of free hydroxyl groups in the C-ring of pancratistatin **2** for apoptosis inducing activity. Finally, attention is also drawn to the weak apoptosis inducing activity of the epoxy-containing derivatives **10** and **11** despite the report of cytotoxicity previously documented for a similar alkaloid (Likhitwitayawuid et al., 1993). We have not ruled out the possibility that non-specific esterases present in hepatoma cells may cleave the acetyl group of derivative **10**, resulting in the generation of an active alkaloid. The weaker activity of the epoxy-containing alkaloid

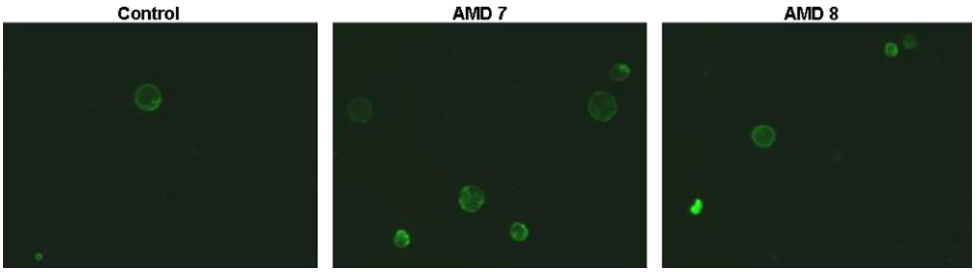


Fig. 4. Annexin-V binding assays were conducted on the 5123tc cells treated with 15 μM of alkaloids **4** and **9**. The flipping of phosphatidyl serine from the inner to outer leaflet of the plasma membrane is a hallmark of apoptosis.

11, which lacks the required C11-hydroxyl group, therefore appears to be unrelated to the apoptosis-inducing pharmacophore possessed by alkaloids **4** and **9**.

3. Concluding remarks

In conclusion, the selective apoptosis inducing activity of Amaryllidaceae alkaloids of the crinane-type is reported and the structural requirements of both the alpha-C2 bridge and a free hydroxyl group at C-11 as pharmacophoric elements is demonstrated. Although this biological activity is reminiscent of the pancratistatin series in certain respects as described above, the existence of a common pharmacophore seems unlikely due to the structural differences and other requirements in the pancratistatin series not found in crinum alkaloids **4** or **9**. The possibility that both Lycorane and Crinane apoptosis-inducing agents act differently on the same or similar biological receptor is however not ruled out by these investigations. These initial results also demonstrate the inspiring selective apoptosis-inducing activity of crinum alkaloids **4** and **9** to animal cancer cells over non-cancerous human cells. Further investigations into the mechanism of apoptosis induction, identification of the target and elaboration of these results to include a wider spectrum of tumour cell types is currently being undertaken in our laboratories.

4. Experimental

4.1. General

Melting points (uncorrected) were measured on a Gallenkamp melting point apparatus. Optical rotations were determined on a Perkin-Elmer 241 polarimeter installed with a λ_{589} sodium lamp. IR spectra were measured on a Bio-Rad FTS-40 series spectrometer in dry film. CIMS were run on a Micromass Quattro Ultima spectrometer fitted with a direct injection probe (DIP) with ionization energy set at 70 eV and HRMS (CI) were performed with a Micromass Q-Tof Ultima spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV 700 spectrometer in $CDCl_3$ with TMS as internal standard, chemical shifts are reported in units of δ (ppm) and coupling constants (J) are expressed in Hz. Silica gel Merck (70-230 mesh) was used for CC, silica gel SIL G/UV_{254} for analyt. and SIL G-25/UV_{254} for prep. TLC (both Macherey-Nagel). Spots on chromatograms were detected under UV light (254 nm) and by Dragendorff's reagent stain.

4.2. Extraction and isolation of alkaloids

Amabiline **5** (Machocho et al., 2004), josephinine **6** (Viladomat et al., 1994, 1997), crinine **7**, crinamine **4** (Campbell et al., 2000), haemanthamine **9** (Herrera et al., 2001), ambelline **8** (Nair et al., 2005), 1,2 β-epoxyambelline **10**, 6α-hydroxyundulatine **11** (Machocho et al., 1999) were all obtained from authenticated plant sources as previously described, voucher specimens have been deposited. All alkaloids were single components by both NMR and thin layer chromatographic analysis.

4.3. Cell models: cancerous and non-cancerous

The rat liver hepatoma (5123tc) cancer cells employed in this study were obtained from Dr. M. Sikorska, IBS, NRC, Ottawa, ON. Cells were grown and maintained in an incubator set at 37 °C, 5% CO_2, and 95% humidity. RPMI-1640 media (Sigma-Aldrich, Oakville, ON) supplemented with 10% fetal bovine serum (FBS) and 10 mg/mL Gentamycin (Life Technologies, Mississauga, ON) was used to culture 5123tc cells.

For the study of non-cancerous cells, rapidly-dividing human embryonic kidney (HEK 293t) cells were purchased from ATCC, Manassas, VA and cultured in DMEM media (Sigma-Aldrich, Oakville, ON) supplemented with 10% FBS, 1% L-glutamine, and 10 mg/mL. These cells were also maintained in an incubator set at 37 °C, 5% CO_2, and 95% humidity.

4.4. Cell treatment

To explore induction of apoptosis by treatment with the various Amaryllidaceae alkaloids, 5123tc cells were grown to 70% confluence and treated for 24, 48, and 72 h with each alkaloid. Various concentrations of the alkaloids diluted in culture media, from a 10 mM stock in DMSO, ranging from 1 to 25 μM were used and apoptotic induction by alkaloid treatment was calculated. In parallel, apoptotic index was also determined in non-cancerous HEK 293t cells treated with the same alkaloids in concentrations ranging from 5 to 20 μM.

4.5. Cellular staining and ED50 assignment

Nuclear morphological changes associated with apoptosis were visualized using 10 μM final concentration of cell-permeable Hoechst 33342 dye (Molecular Probes, Eugene, OR) in 5123tc and HEK293t cells. Following treatment with various alkaloid concentrations, over multiple treatment times, Hoechst 33342 dye was added directly to culture media and allowed to incubate for 10 min at 37 °C. Cells were examined using a fluorescent microscope (Leica DM IRB, Germany); pictures were taken at 40×. To obtain an apoptotic index, brightly stained condensed nuclei (apoptotic cells) were counted in a minimum of 5 fields, each containing more than 100 cells, so that for each data point, a minimum of 500 total cells were counted. Data was calculated and an apoptotic index was created such that apoptotic cells were expressed as a percentage of the total number of cells counted. The ED50, or effective dosage defined as the concentration which induces apoptosis in 50% of cells after 72 h of treatment, was calculated from

the slope of the curve displaying the apoptotic index of the particular alkaloids at this time-point. Statistical analysis was performed using STATISTICA, from the data of three different experiments and $p < 0.05$ was obtained for alkaloids **4** and **9**.

4.6. Synthesis and characterization of derivatives *12* and *13*

4.6.1. Synthesis of 11-*O*-acetoxycrinamine *12*

Crinamine **4** (5.0 mg, 16.6 μmol) and 4-*N*,*N*-dimethylaminopyridine (DMAP) (0.2 mg, 1.66 μmol) were dissolved at r.t. in CH_2Cl_2 (200 μl) to which pyridine (2.0 μl, 33.2 μmol) was added. After 15 min, Ac_2O (4.0 μl, 33.2 μmol) was introduced and the solution stirred until TLC (10% EtOAc/Hex) indicated the reaction to be complete (~2 h). The mixture was then diluted with H_2O (2 ml) and extracted with CH_2Cl_2 (3 × 2 ml), with the combined organic fractions dried over (anhydrous Na_2SO_4) and the solvent removed under reduced pressure to yield a gum which was purified by prep. TLC (10% EtOAc/Hex) to give 11-*O*-acetoxycrinamine **12** (5.6 mg, 98%) as a white crystalline powder. M.p. 130–132 °C. $[\alpha]_D^{25}+92(CHCl_3, c0.1)$. IR υ_{MAX} cm^{-1} (NaCl): 2363, 1738, 1718 (C═O), 1653 (Ph), 1459, 1236, 1039 (C–O), 933 (OCH_2O). HRMS (CI): calcd 344.1498 for $C_{19}H_{22}NO_5$, found 344.1508. CIMS 70 eV, *m/z* (rel. int.): 344 $[M + 1]^+$ (100), 312 (5), 284 $[M + 1\text{-}OAc]^+$, 268 $[M+1\text{-}OAc\text{-}CH_3]^+$, 252 (25), 223 (30), 211 (15), 181 (5). ^1H NMR (700 MHz, $CDCl_3$): δ 1.92 (1H, *ddd*, *J* = 12.6, 6.4, 4.2 Hz, H-4 β), 2.02 (3H, *s*, O–CO–C*H*$_3$), 2.12 (1H, *ddd*, *J* = 13.3, 12.6, 9.2 Hz, H-4α), 3.21 (1H, *dd*, *J* = 13.3, 4.2 Hz, H-4a), 3.39 (1H, *dd*, *J* = 14.0, 4.2 Hz, H-12*exo*), 3.40 (1H, *dd*, *J* = 14.0, 6.3 Hz, H-12*endo*), 3.41 (3H, *s*, 3-OC*H*$_3$), 3.72 (1H, *d*, *J* = 16.8 Hz, H-6α), 3.98 (1H, *ddd*, *J* = 9.2, 6.4, 2.1 Hz, H-3), 4.34 (1H, *d*, *J* = 16.8 Hz, H-6 β), 4.96 (1H, *dd*, *J* = 6.3, 4.2 Hz, H-11), 5.90 (2H, 2*d*, *J* = 1.4 Hz, –OC*H*$_2$O–), 6.00 (1H, *d*, *J* = 10.5 Hz, H-1), 6.15 (1H, *dd*, *J* = 10.5, 2.1 Hz, H-2), 6.47 (1H, *s*, H-7), 6.87 (1H, *s*, H-10). ^{13}C NMR (176 MHz, $CDCl_3$): δ 21.5 (q, O–CO–*C*H$_3$), 30.0 (*t*, C-4), 49.6 (*s*, C-10b), 55.9 (*q*, 3-OC*H*$_3$), 60.5 (*t*, C-12), 61.2 (*t*, C-6), 66.5 (*d*, C-4a), 76.2 (*d*, C-3), 80.5 (*d*, C-11), 101.1 (*t*, O*C*H$_2$O), 104.0 (*d*, C-10), 106.8 (*d*, C-7), 124.0 (*d*, C-2), 126.4 (*s*, C-6a), 133.3 (*d*, C-1), 134.7 (*s*, C-10a), 146.7 (*s*, C-8), 146.9 (*s*, C-9), 172.7 (*s*, O–*C*O–CH$_3$).

4.6.2. Synthesis of 11-*O*-(4-methoxybenzoyl)crinamine *13*

Crinamine **4** (5.0 mg, 16.6 μmol) and DMAP (0.2 mg, 1.66 μmol) were dissolved at r.t. in CH_2Cl_2 (200 μl) to which pyridine (2.0 μl, 33.2 μmol) was added. After 15 min, 4-methoxybenzoyl chloride (4.0 μl, 33.2 μmol) was introduced and the solution stirred at 40 °C until TLC (10% EtOAc/Hex) indicated the reaction to be complete (~4 h). The mixture was then diluted with H_2O (2 ml) and extracted with CH_2Cl_2 (3 × 2 ml), with the combined organic fractions dried (anhydrous Na_2SO_4) and the solvent removed under reduced pressure to yield a gum which was purified by prep. TLC (10% EtOAc/Hex) to give 11-*O*-(4-methoxybenzoyl)crinamine **13** (7.0 mg, 97%) as a white crystalline powder. M.p. 118–120 °C. $[\alpha]_D^{25}+113$ $(CHCl_3, c0.1)$. IR υ_{MAX} cm^{-1} (NaCl) 2927, 1707 (C═O), 1606 (Ph), 1508, 1483, 1256, 1102, 1034 (C–O), 937 (OCH_2O). HRMS (CI): calcd 436.1760 for $C_{25}H_{26}NO_6$, found 436.1755. CIMS 70 eV, *m/z* (rel. int.): 436 $[M + 1]^+$ (73), 406 $[M + 1\text{-}2CH_3]^+$ (12), 344 (54), 300 $[M + 1\text{-}O(CO)ArOCH_3]^+$ (16), 284 (50), 269 (40), 252 (26), 224 (27), 210 (28), 135 $[O(CO)ArOCH_3]^+$ (100). ^1H NMR (700 MHz, $CDCl_3$): δ 2.07 (1H, *ddd*, *J* = 12.4, 6.4, 3.5 Hz, H-4 β), 2.21 (1H, *ddd*, *J* = 13.3, 12.4, 9.2 Hz, H-4α), 3.29 (1H, *dd*, *J* = 13.3, 3.5 Hz, H-4a), 3.39 (3H, *s*, 3-OC*H*$_3$), 3.50 (1H, *dd*, *J* = 14.7, 3.5 Hz, H-12*exo*), 3.54 (1H, *dd*, *J* = 14.7, 7.0 Hz, H-12*endo*), 3.78 (1H, *d*, *J* = 16.8 Hz, H-6α), 3.86 (3H, *s*, Ar–OC*H*$_3$), 4.01 (1H, *ddd*, *J* = 9.2, 6.4, 2.8 Hz, H-3), 4.39 (1H, *d*, *J* = 16.8 Hz, H-6 β), 5.18 (1H, *dd*, *J* = 7.0, 3.5 Hz, H-11), 5.91 (2H, 2*d*, *J* = 1.4 Hz, –OC*H*$_2$O–), 6.00 (1H, *d*, *J* = 10.5 Hz, H-1), 6.22 (1H, *dd*, *J* = 10.5, 2.8 Hz, H-2), 6.51 (1H, *s*, H-7), 6.90 (1H, *s*, H-10), 6.91 (2H, *d*, *J* = 9.5 Hz, H-4′/H-6′), 7.98 (2H, *d*, *J* = 9.5 Hz, H-3′/H-7′). ^{13}C NMR (176 MHz, $CDCl_3$): δ 30.6 (*t*, C-4), 49.5 (*s*, C-10b), 55.9 (*q*, 5′-O*C*H$_3$), 56.0 (*q*, 3-O*C*H$_3$), 61.2 (*t*, C-12), 61.3 (*t*, C-6), 66.6 (*d*, C-4a), 76.2 (*d*, C-3), 80.6 (*d*, C-11), 101.1 (*t*, –O*C*H$_2$O–), 104.0 (*d*, C-10), 106.9 (*d*, C-7), 113.8 (2*d*, H-4′/H-6′), 122.6 (*s*, C-2′), 124.1 (*d*, C-2), 126.5 (*s*, C-6a), 131.9 (2*d*, C-3′/C-7′), 133.2 (*d*, C-1), 134.9 (*s*, C-10a), 146.7 (*s*, C-8), 147.0 (*s*, C-9), 163.6 (*s*, C-5′), 165.7 (*s*, O–*C*O–Ar).

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