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Evaluation of the Effects of Several Zoanthamine-type Alkaloids on the Aggregation of Human Platelets

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Abstract—Ten zoanthamine-type alkaloids from two marine zoanthids belonging to the *Zoanthus* genus (*Zoanthus nymphaeus* and *Zoanthus* sp.) along with one semisynthetic derivative were evaluated for their antiplatelet activities on human platelet aggregation induced by several stimulating agents. 11-Hydroxyzoanthamine (**11**) and a synthetic derivative of norzoanthamine (**16**) showed strong inhibition against thrombin-, collagen- and arachidonic acid-induced aggregation, zoanthenol (**15**) displayed a selective inhibitory activity induced by collagen, while zoanthaminone (**10**) behaved as a potent aggregant agent. These evaluations allowed us to deduce several structure–activity relationships and suggest some mechanisms of action for this type of compounds.

Introduction

Marine zoanthids belonging to the genus *Zoanthus* (Phylum Cnidaria, Class Anthozoa, Order Zoanthidea, Family Zoanthidae) are able to biosynthesize a family of nitrogen marine metabolites known as zoanthamine alkaloids. Zoanthamine (**1**) was isolated by Faulkner et al. in 1984 from an unidentified colonial zoanthid *Zoanthus* sp. collected at the Visakhapatnam coast of India and this compound was identified as the first member of a new class of alkaloids. The structure of **1** was determined by X-ray diffraction, which revealed that this new molecule was unrelated to any previously known alkaloid structure.[1] Over the past few years, more than 12 of these compounds have been reported.[2] They are characterized not only by their unique array of structural and stereochemical complexity, but also by their important and interesting pharmacological activities. For example, zoanthenamide (**2**), zoanthamide (**3**), and 28-deoxyzoanthenamide (**4**) showed inhibition of phorbol myristate acetate (PMA)-induced inflammation in mouse ear.[3,4] Furthermore, norzoanthamine (**5**), norzoanthaminone (**6**), epinorzoanthamine (**7**), and oxyzoanthamine (**8**) were found to inhibit the growth of P-388 murine leukemia cell lines, with IC_{50} values of 24, 1.0, 2.6, and 7.0 μg/mL, respectively.[5] Norzoanthamine (**5**) has also been reported as a promising candidate for an osteoporotic drug as an IL-6 inhibitor.[6]

Platelet aggregation plays a crucial role in physiological hemostasis and pathological thrombosis. In fact, platelet thrombus formation is implicated in both venous thrombosis, like pulmonary embolism, deep vein thrombosis, disseminated intravascular coagulation, and, to a greater extent, in arterial thrombosis, that is myocardial infarction, stroke, and other cardiovascular diseases. Moreover, it is clear today that platelet deposition on the arterial wall significantly contributes to the onset and progress of atherosclerotic lesions, which may result in the occlusion of the vessel.[7] The general acceptance of these facts has drastically changed antithrombotic strategies: antiplatelet drugs, and specifically inhibitors of platelet aggregation, have become essential tools in the therapy of arterial thrombotic disorders.[8]

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For this reason, the search for new antiplatelet drugs that are more effective and specific is a priority in basic and clinical pharmacological investigation.

In our continuing search for active metabolites from marine sources and, more specifically, natural products with inhibitory effects on human platelet aggregation,[9] we reported previously a preliminary study of several zoanthamine-type alkaloids isolated from the colonial zoanthid *Zoanthus nymphaeus*. We found a strong specific inhibitory activity for 3-hydroxynorzoanthamine (**9**) on human platelet aggregation induced by several stimulating agents, while zoanthaminone (**10**) behaved as a potent activating agent and zoanthamine (**1**) lacked activity in this assay.[10]

Results

One of our aims was to explore the structure–activity relationships of this family of compounds as antiplatelet agents. With this goal in mind, we recently reported the isolation and structural determination of six new (**9** and **11**–**15**) and five known (**1**, **5**, **6**, **8**, and **10**) zoanthamine-type alkaloids from specimens of *Zoanthus* sp. collected around the Canary Islands[11] and have performed a biological assay in vitro of the pure isolated compounds, focusing on the human platelet aggregation induced by several stimulating agents. In this paper we report the antiplatelet activity of ten of these natural zoanthamine-type alkaloids and one semisynthetic derivative (**16**), as well as a number of structure–activity relationships deduced from the data obtained (Chart 1).

The zoanthamine-type alkaloids evaluated in this study were isolated and identified from the crude extracts of two marine zoanthids belonging to *Z. nymphaeus* and an unidentified species of *Zoanthus* sp.

The colonial zoanthid *Z. nymphaeus* occurs as dense mats on intertidal rocks on the coast of Salvador de Bahia (Brazil). This species ejects water when disturbed and can cause irritation if the water reaches the victim's eyes. This phenomenon was found in another *Zoanthus* species from the coast of India.[3] The organic extracts of this organism were submitted to separation to give a zoanthamine mixture, the components of which—after purification and structure elucidation—were identified as zoanthamine (**1**), 3-hydroxynorzoanthamine (**9**), and zoanthaminone (**10**).[10]

The second zoanthid was an unidentified species of *Zoanthus* collected in the intertidal zone at Punta Hidalgo, Bajamar at Tenerife in the Canary Islands (Spain). From the crude extracts of this organism we isolated and identified zoanthamine (**1**), norzoanthamine (**5**), norzoanthaminone (**6**), oxyzoanthamine (**8**), 3-hydroxynorzoanthamine (**9**), zoanthaminone (**10**), 11-hydroxyzoanthamine (**11**), 11-hydroxynorzoanthamine (**12**), 30-hydroxynorzoanthamine (**13**), epioxyzoanthamine (**14**) and zoanthenol (**15**). A synthetic derivative, compound **16**, was also obtained by methylation of norzoanthamine (**5**) with CH_2N_2.[11]

We studied the in vitro effects of the alkaloids **1**, **5** and **8**–**16** on washed human platelet aggregation. Aggregation was evaluated by a turbidimetric method[12] and was defined as the change in light transmission. Firstly, we evaluated the pro-aggregant activity of all the alkaloids for 5 min after their introduction into the aggregometer cuvette. Secondly, we investigated the antiplatelet effects of the alkaloids and aspirine on the aggregation induced by either thrombin (0.075 UI/mL), collagen (12.5 μg/

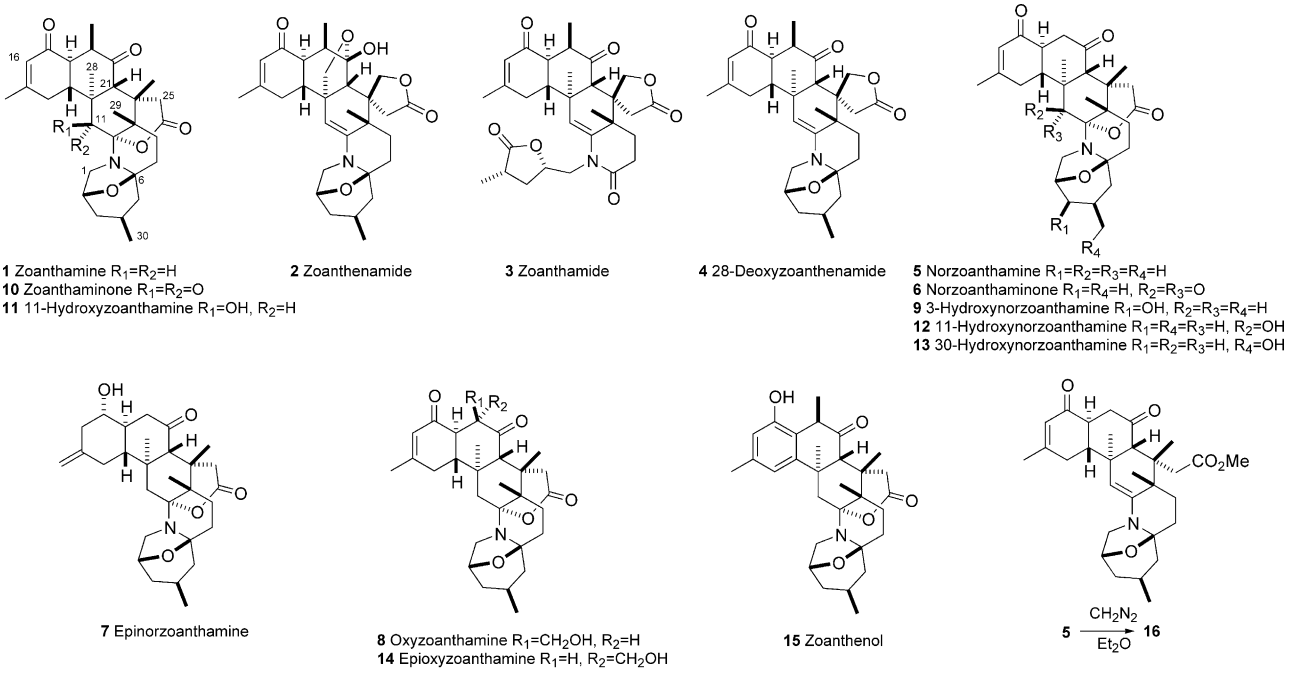


Chart 1.

mL) or arachidonic acid (15 μM). With these platelets inducers we could obtain valuable information about effects of zoanthamine-type alkaloids on thrombin receptor/phospholipase C pathway, collagen receptor/phospholipase A_2 pathway, and cyclooxygenase/thromboxane A_2 pathway. The small amount of alkaloids available did not allow us to work with other major platelets inducers (for example ADP) and study more signal transduction pathways. The results are shown in Figures 1 and 2.

Aspirine, at the concentration of 0.25 mM, was used as a reference control. It completely abolished arachidonic acid-induced platelet aggregation, which is totally dependent on thromboxane A_2 synthesis by cyclooxygenase, and inhibited collagen (83±5%, $n=4$) and thrombin (58±7%, $n=4$) induced aggregation, which are partially dependent on thromboxane synthesis.

In this study zoanthamines behaved as a heterogeneous group of compounds in terms of their effect on platelet reactivity, both at rest and with activated platelets. This fact indicates the importance of small structural variations for the biological activity of the zoanthamines and suggests a specific action of the active compounds.

A subgroup of five alkaloids, namely 3-hydroxynorzoanthamine (**9**), 11-hydroxynorzoanthamine (**12**), 30-hydroxynorzoanthamine (**13**), 11-hydroxyzoanthamine (**11**), and the synthetic derivative of norzoanthamine **16**, showed a clear inhibitory activity on the aggregation induced by all three stimulating agents employed. However, there were marked differences in the activity profiles among these compounds. On the one hand, compounds **11** (0.3, 0.5 and 1 mM) and **16** (0.3, 0.5 and 1 mM) strongly inhibited platelet aggregation induced by thrombin, collagen, and arachidonic acid. Indeed, these compounds almost completely inhibited the aggregant responses at the highest concentrations used (Fig. 1A). On the other hand, compounds **9** (0.125, 0.5 and 1 mM), **12** (0.3 and 1 mM) and **13** (0.3 and 1 mM) showed a more selective effect towards the aggregations induced by collagen and arachidonic acid. More specifically, at a concentration of 1 mM compound **12** completely inhibited collagen- and arachidonic acid-induced aggregation, while compound **9** caused more than 80% inhibition of aggregation induced by both agents. In contrast, thrombin-induced aggregation was reduced by compounds **12** and **9**, also at a concentration of 1 mM, by 52 and 24%, respectively. Although compound **13** showed the same profile, it behaved as a less powerful antiplatelet agent (Fig. 1B). Overall, these results suggest a mode of action (exclusive or additive to others) for this group of compounds on a common and essential step in the platelet activation. Such steps could include stimulation of calcium input into the cell, activation of protein kinase C (PKC) or diminution of intracellular AMPc levels. It is necessary to bear in mind the key participation of thromboxane A_2 (TXA_2) in the aggregation process,

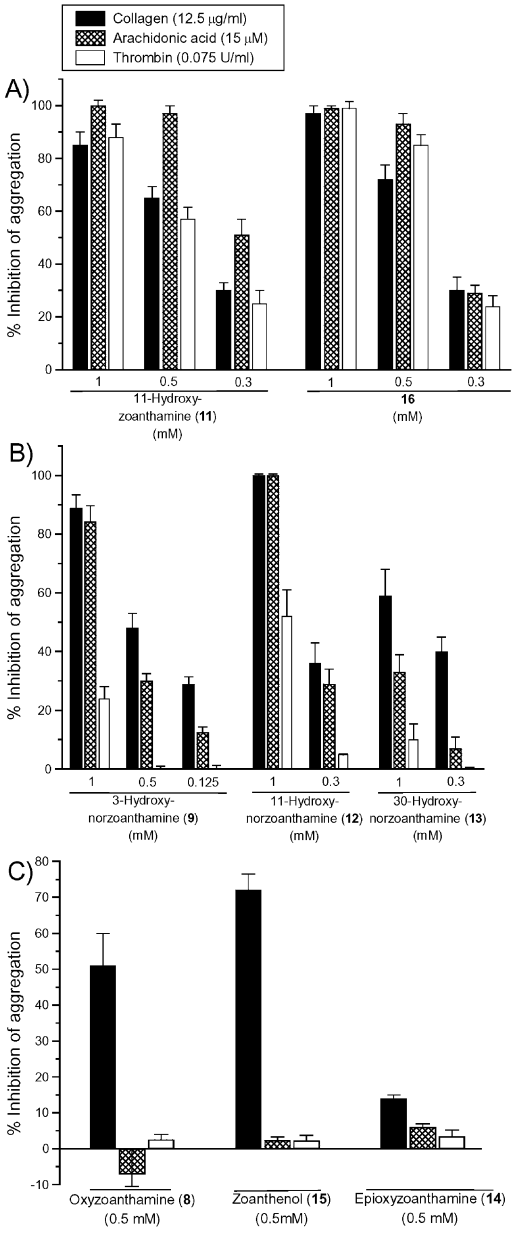


Figure 1. Effects of (A) 11-hydroxyzoanthamine (**11**) and **16**, (B) 3-hydroxynorzoanthamine (**9**), 11-hydroxynorzoanthamine (**12**), and 30-hydroxynorzoanthamine (**13**); and (C) oxyzoanthamine (**8**), zoanthenol (**15**), and epioxyzoanthamine (**14**) at different concentrations on washed human platelet aggregation induced by collagen, arachidonic acid and thrombin. Each value is the mean of 2–4 replicate assays. Vertical bars show standard errors of the mean.

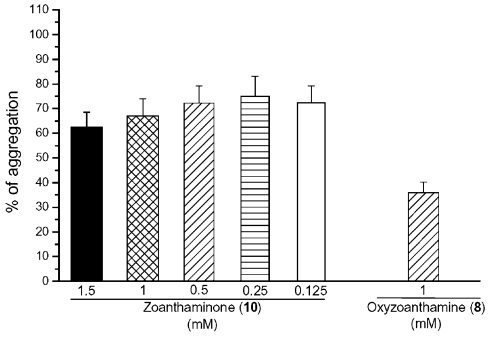


Figure 2. Aggregant effects induced by zoanthaminone (**10**) and oxyzoanthamine (**8**) on resting human platelets. Each value is the mean of 2–4 replicate assays. Vertical bars show standard errors of the mean.

especially when platelet aggregation is induced by arachidonic acid (via direct metabolism to TXA_2) or collagen (via activation of phospholipase A_2-PLA_2-). Therefore, the inhibition of TXA_2 synthesis or the blockage of its receptors could be involved in the anti-aggregant responses of this group of zoanthamines, especially in the cases of compounds **9**, **12**, and **13**.

Another series of zoanthamines behaved as selective inhibitors of collagen-induced platelet aggregation, causing little or no effect against the aggregation induced by either arachidonic acid or thrombin. This is the case for compounds **15** (zoanthenol; 72% inhibition at 0.5 mM), **8** (oxyzoanthamine; 50% inhibition at 0.5 mM), and **14** (epioxyzoanthamine; 14% inhibition at 0.5 mM), as shown in Figure 1C. The activity profile observed suggests that these compounds could specifically interfere with an essential step in collagen-induced platelet activation—for example through antagonism of its receptor. The fact that compound **8** caused a pro-aggregant effect on increasing its concentration up to 1 mM (see Fig. 2) suggests that this zoanthamine could act as a partial agonist on the collagen receptor.

Oxyzoanthamine (**8**) at a concentration of 1 mM (as already discussed) and zoanthaminone (**10**) at concentrations in the range 1.5–0.125 mM showed an intense, slow-onset, and irreversible pro-aggregant effect when they were added to the platelet suspension at rest. In all cases the pro-aggregant activity exceeded 50% of the response evoked by thrombin at 0.075 UI/mL, one of the more powerful platelet agonists (Fig. 2).

Finally, neither compound **1** (zoanthamine; 1 mM) nor **5** (norzoanthamine; 1 mM) caused significant aggregation when added to resting platelets, nor did they inhibit the aggregation induced by collagen, arachidonic acid or thrombin.

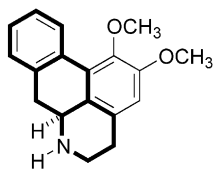
Structure–activity relationships

This pharmacological study indicates that significant effects are in operation in terms of the influence of structures and substituents on the activity levels, because small changes in zoanthamine structures have marked repercussions on the activity. A number of general observations can be made on the basis of the data shown in Figures 1 and 2:

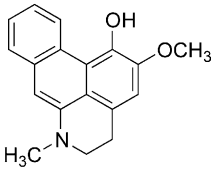
1. The presence of an additional hydroxyl group in some of the compounds attracted our attention because, firstly, the introduction of this group at C-11 turns the inactive compound zoanthamine (**1**) into the most active compound 11-hydroxyzoanthamine (**11**). This compound showed total inhibition of platelet aggregation induced by collagen, AA and thrombin (Fig. 1A). Furthermore, a similar effect is observed with norzoanthamine (**5**), an inactive compound in terms of platelet aggregation that becomes both active and selective with the presence of an additional hydroxyl group at C-3 (**9**), C-30 (**13**) or, more markedly, at C-11 (**12**). In the latter case, the inhibition at the highest concentration is total against collagen and AA-induced aggregation, suggesting the importance of that position in controlling the induction of activity (Fig. 1B).
2. When carbon C-26 appears in an oxidised form, that is in oxyzoanthamine (**8**) or epioxyzoanthamine (**14**), an antiplatelet aggregation activity is generated in relation to zoanthamine (**1**) (Fig. 1C).
3. The absence of the methyl group C-26 led to an increase in the selective inhibitory action in some cases, as it can be observed in the selective effect against collagen- and AA-induced aggregation found for 11-hydroxynorzoanthamine (**12**), 3-hydroxynorzoanthamine (**9**), and 30-hydroxynorzoanthamine (**13**) in comparison to those of 11-hydroxyzoanthamine (**11**) (Fig. 1A and B).
4. An additional degree of unsaturation between C-10/C-11, present in the very active semisynthetic derivative of norzoanthamine (**16**), turns the inactive norzoanthamine (**5**) into one of the most active derivative of this series (Fig. 1A).
5. The degree of oxidation around the A and B rings on the zoanthamine framework can modulate this antiplatelet effect. Thus, a selective inhibitory effect against collagen-induced aggregation is observed in the case of **8**. A similar and strong selective activity was observed for zoanthenol (**15**), which possesses aromatization in ring A (Fig. 1C).
6. In contrast to the inhibitory effects described above, oxyzoanthamine (**8**) and zoanthaminone (**10**) gave rise to irreversible platelet aggregation (Fig. 2). The oxidation state of carbons C-11 and C-26 in these compounds, in relation to zoanthamine, reinforce the importance of these positions for bioactivity.

In order to establish additional structure–activity relationships between these compounds, an approximation was made by drawing common elements from other compounds such as aporphines, which were recently reported as strong inhibitors of platelet aggregation of washed rabbit platelets induced by ADP, AA, collagen and PAF (platelet-activating factor).[13] Such a comparison with the strong inhibitor (+)-nornuciferine (**17**) allowed us to identify a similar fragment in the carbon backbone of our zoanthamine series, as shown in Chart 2. Moreover, the strong active aporphinoid 7-dehydrolirinidine (**18**) is particularly reminiscent of compound **16** (Chart 2).

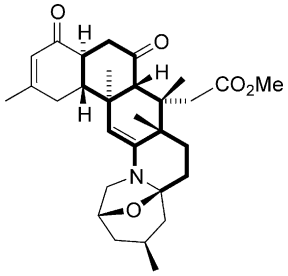
In summary, the evaluation of a series of nitrogen marine metabolites known as zoanthamine alkaloids, obtained from two marine zoanthids [*Zoanthus* sp. from Canary Islands (Spain) and *Z. nymphaeus* from Salvador de Bahia (Brasil)], on human platelet aggregation induced by several stimulating agents showed that it is possible to induce biological activity by small and



17 (+)-Nornuciferine



18 7-Dehydroliridine



16

Chart 2.

selective changes in this type of structure. The results indicate that the introduction of one oxidized function in combination with the presence or absence of the methyl group C-26 in this class of alkaloids are two of the fundamental factors related to the modulation of the inhibitory platelet aggregation induced because the most active zoanthamine-type alkaloids have this position in an oxidized form: compounds **11** and **16** are active agents against aggregation induced by all three stimulating agents, compound **12** against collagen- and AA-induced aggregation, and compound **10** is a potent aggregant agent. Finally, this study confirms the utility of the zoanthamine-type alkaloids as good model systems for rational drug design.

Experimental

Chemistry

General. Optical rotations were determined on a Perkin–Elmer 241 polarimeter. IR spectra were measured on a Bruker IFS55 spectrometer. The NMR spectra were obtained with a Bruker AVANCE 500 MHz instrument. Chemical shifts are reported relative to TMS and coupling constants are given in Hz. HRMS was performed on a VG AutoSpec FISON spectrometer. HPLC was carried out with an LKB 2248 system equipped with a differential diffractometer detector. Si gel CC and TLC were performed on Si gel Merck 60 G. TLC plates were visualised by spraying with Dragendorff's reagent and heating. All solvents were purified by standard techniques.

Isolation and chromatographic separation. The zoanthid *Z. nymphaeus* (Phylum Cnidaria, Class Anthozoa, Order Zoanthidea, Family Zoanthidae) was collected by hand on intertidal rocks at Pituba beach in Salvador de Bahia (Brazil) in September 1997. Polyps were identified by Prof. S. Peixinho at the Departamento de Zoologia, Instituto de Biologia, Universidade Federal da Bahía, Brazil. A voucher specimen (BR 9701) was deposited at the Departamento de Química Fundamental, Universidade de A Coruña. A picture of the specimen can be obtained from the authors. Specimens of the zoanthid (1 kg, dry wt) were soaked in MeOH at room temperature for 48 h (3×500 mL). The extracts were combined and the solvents were removed under reduced pressure. The residue was subjected to solvent partitioning as described previously to give hexane (4.56 g), CH_2Cl_2 (1.79 g), and *n*-BuOH (13.98 g) soluble fractions. The fraction that was soluble in CH_2Cl_2 was fractionated by GPC on Sephadex LH-20 (eluted with MeOH). The fractions containing the zoanthamine mixture were combined, the solvent evaporated under reduced pressure and the residue purified by flash chromatography on Si gel using CH_2Cl_2 and EtOAc (0–100%) mixtures as eluents, with a final wash using increasing amounts of MeOH. Further purification by flash chromatography on Si gel EtOAc/MeOH and by normal-phase HPLC with EtOAc/MeOH (95:5), gave zoanthaminone (10) (3.5 mg) and zoanthamine (1) (46 mg), while reversed-phase HPLC with MeOH/H_2O (85:15) was used to obtain 3-hydroxynorzoanthamine (9) (10 mg).

Samples of *Zoanthus* sp. were collected in March 1998 in the intertidal zone at Punta Hidalgo, Bajamar (Tenerife, Canary Islands, Spain). Polyps were identified by Prof. A. Brito at the Departamento de Biología Marina, Universidad de La Laguna, Tenerife. The fresh material (0.75 kg) was extracted with 1:1 acetone/methanol for 24 h at room temperature. The combined extracts were evaporated in vacuo to leave a dark-green viscous oil (20 g, 3.8% dry weight). The crude extract was chromatographed on Sephadex LH-20 and a medium pressure silica gel chromatography column using *n*-hexane/$CHCl_3$/MeOH (2:1:1) and $CHCl_3$/MeOH (95:5) as eluents, respectively. Fractions exhibiting similar TLC profiles with Dragendorff's reagent were selected and each one was rechromatographed on a medium pressure reversed-phase Lobar LiChropred RP-8 column with MeOH/H_2O (85:15) and, subsequently, on a μ-Bondapack reversed-phase C-18HPLC column using CH_3CN/MeOH/H_2O in different proportions. This final purification, carried out by HPLC, yielded pure products zoanthamine (**1**) (10.5 mg), norzoanthamine (**5**) (36 mg), norzoanthaminone (**6**) (3.6 mg), oxyzoanthamine (**8**) (5.4 mg), 3-hydroxynorzoanthamine (**9**) (1.1 mg), zoanthaminone (**10**) (2.8 mg), 11-hydroxyzoanthamine (**11**) (2.8 mg), 11-hydroxynorzoanthamine (**12**) (5.2 mg), 30-hydroxynorzoanthamine (**13**) (3.8 mg), epioxyzoanthamine (**14**) (2.6 mg) and zoanthenol (**15**) (1.6 mg).

Compound 16. To a stirred solution of norzoanthamine (**5**) (10 mg, 0.02 mmol) in diethyl ether (1 mL) was added dropwise a solution of CH_2N_2 (0.1 mmol) in ether. The reaction mixture was stirred at rt for 10 min. The solvent was evaporated in vacuo and subsequent flash chromatography gave compound **16** in quantitative yield.

Platelet aggregation

Materials. The reagents used in this study were purchased from the following sources: thrombin and arachidonic acid from Sigma Chemical Co. (St. Louis, USA) and collagen type I from Chrono-Log Corp. (Havertown, USA). Metrizamide was obtained from Nycomed Pharma AS (Oslo, Norway). All other reagents used were of analytical grade. Thrombin and arachidonic acid were prepared daily in de-ionized water from stock solutions kept a −20 °C; collagen was kept at 4 °C and used directly as supplied. Metrizamide was dissolved in de-ionized water (35%) and kept at 4 °C with protection from daylight. Stock solutions (50 mM for compound 5 and 100 mM for compounds **1** and **8**–**16**) were prepared in acid de-ionized water (compound **1**), methanol (**5**, **8**, **9** and **16**), DMSO (**10**, **11**, **12**, **13** and **14**) and absolute ethanol (**15**) and were kept at 4 °C. The concentrations of vehicles present in the platelet suspension (<1%) did not modify significantly the responses elicited by the stimulating agents (thrombin, collagen or arachidonic acid).

Assays. Human platelets were obtained from a platelet concentrate from healthy drug-free donors. Anticoagulation was induced with ACD (13 mM citric acid, 12.4 mM trisodium citrate and 11 mM glucose). Platelets were isolated from the concentrate by centrifugation at 1000 g (22 °C, 10 min) in a discontinuous metrizamide gradient (25–10%). The material was washed by centrifugation, under the same conditions, in a pH 6 buffer (140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose). The washed platelets were resuspended in a modified Tyrode–HEPES buffer pH 7.4 (140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES, 10 mM glucose) at a final concentration of 300,000 platelets/mL. Calcium chloride was added to the suspension one h before the beginning of the assays in order to achieve a final concentration of 2 mM.

Platelet aggregation was measured by the turbidimetric method of Born and Cross[12] using a dual channel aggregometer (Chrono-Log Co., USA). Aliquots (400 μL) of the washed platelet suspension were pre-incubated at 37 °C for 5 min under constant stirring (1100 rpm); 4 μL of the appropriate compound or the vehicle alone were then added. After a 5-min incubation period, the stimulating agent was added (where necessary). The platelet activators used were thrombin 0.075 UI/mL, collagen 12.5 μg/mL or arachidonic acid 15 μM. Maximal aggregation response induced by the stimulating agents was defined as the maximum change in light transmission over 5 min in the presence of vehicle without the compounds. Antiaggregant activity was established as the percentage reduction of this response in the presence of the compounds. Pro-aggregant activity was defined as the percentage change in light transmission induced by the compounds without addition of any platelet activator.

Acknowledgements

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