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Zebrafish Bioassay-Guided Microfractionation Identifies Anticonvulsant Steroid Glycosides from the Philippine Medicinal Plant *Solanum torvum*

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ABSTRACT: Medicinal plants used for the treatment of epilepsy are potentially a valuable source of novel antiepileptic small molecules. To identify anticonvulsant secondary metabolites, we performed an in vivo, zebrafish-based screen of medicinal plants used in Southeast Asia for the treatment of seizures. *Solanum torvum* Sw. (Solanaceae) was identified as having significant anticonvulsant activity in zebrafish larvae with seizures induced by the $GABA_A$ antagonist pentylenetetrazol (PTZ). This finding correlates well with the ethnomedical use of this plant in the Philippines, where a water decoction of *S. torvum* leaves is used to treat epileptic seizures. HPLC microfractionation of the bioactive crude extract, in combination with the in vivo zebrafish seizure assay, enabled the rapid localization of several bioactive compounds that were partially identified online by UHPLC-TOF-MS as steroid glycosides. Targeted isolation of the active constituents from the methanolic extract enabled the complete de novo structure identification of the six main bioactive compounds that were also present in the traditional preparation. To partially mimic the in vivo metabolism of these triterpene glycosides, their common aglycone was generated by acid hydrolysis. The isolated molecules exhibited significant anticonvulsant activity in zebrafish seizure assays. These results underscore the potential of zebrafish bioassay-guided microfractionation to rapidly identify novel bioactive small molecules of natural origin.

KEYWORDS: *Zebrafish, HPLC microfractionation, Solanum torvum, Solanaceae, saponins, antiepileptic activity*

1. INTRODUCTION

Epilepsy is a common neurological disorder characterized by the manifestation of spontaneous seizures as a result of abnormal neuronal activity in the brain. Currently known factors linked to the emergence of epilepsy are stroke, brain injury, brain tumors, and infections such as meningitis, but in 70% of cases the cause is not known. Epilepsy affects around 65 million people worldwide, especially children, and has a higher prevalence in tropical countries, particularly in Africa.[1,2]

Currently, there are a large number of antiepileptic drugs (AEDs), each of them being preferentially used depending on the type of seizure. Importantly, however, almost 30% of patients suffering from epilepsy remain resistant to these existing treatments.[3] Many currently available AEDs also exhibit serious neurological side effects such as depression, cognitive impairment, and sedation, in addition to other drug-induced toxicities such as gastrointestinal distress and hepatotoxicity.[4−7] For these different reasons, there is a clear need to identify new AEDs with efficacy against pharmacoresistant epilepsy and ideally with minimal or no adverse effects.

The identification of new AEDs is a significant challenge due to the heterogeneity and complexity of epileptic seizures. The evaluation of antiepileptic drug leads requires numerous in vitro and in vivo models which mimic the different mechanisms of action involved in epilepsy.[8] In several in vivo assays, epilepsy is induced after administration of chemical proconvulsants such as

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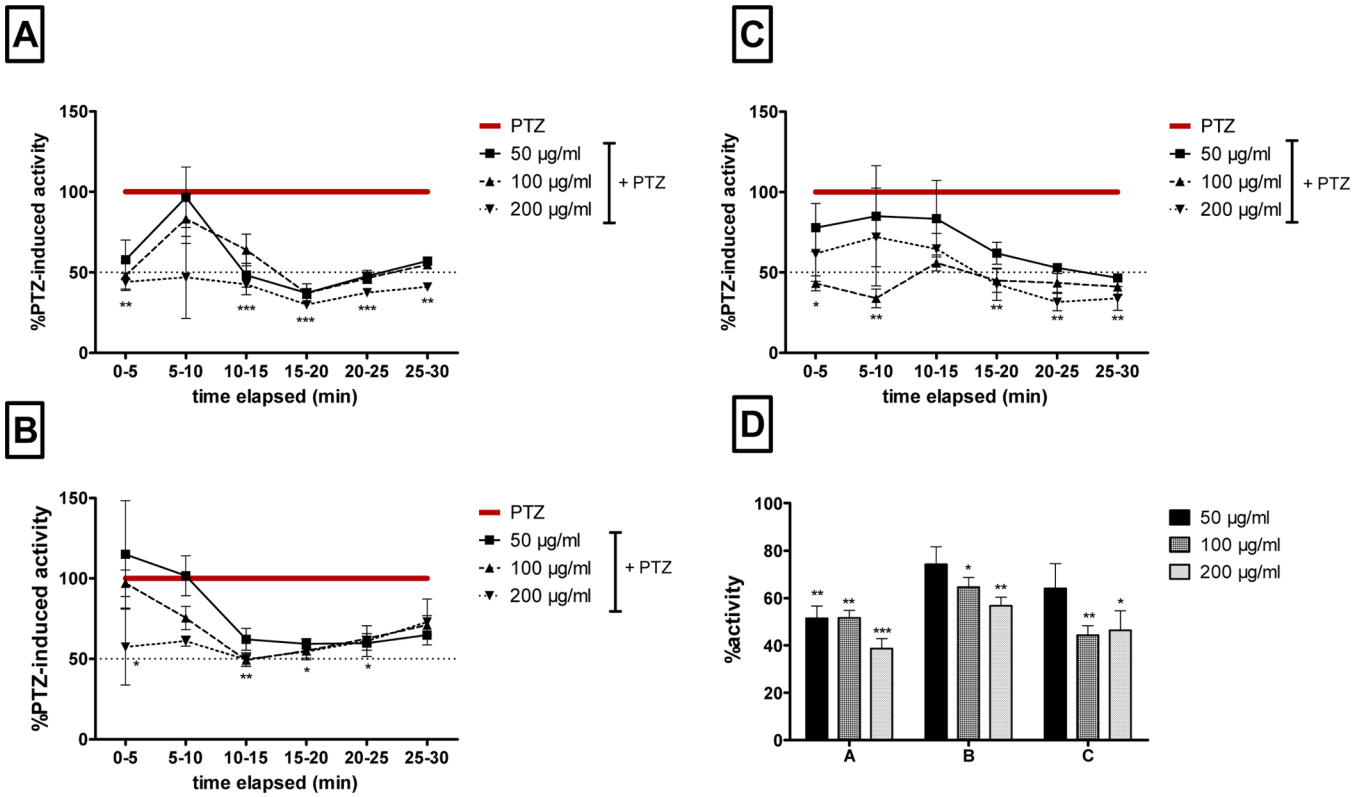

Figure 1. (A–C) PTZ-induced activity curve for a 30 min tracking period of 7 dpf zebrafish larvae after 18 h pretreatment with different concentrations of (A) *S. torvum* aqueous extract (from serial extractions of increasing polarity), (B) *S. torvum* methanol crude extract, and (C) *S. torvum* decoction. (D) Total movement after 30 min tracking period (expressed in %activity) of (A)–(C), represented in a bar graph. All results were normalized against PTZ controls (set at 100%). Analysis of the activity curves was done by two-way ANOVA, with *P* values of <0.05 (*), < 0.01 (**), and <0.001 (***) indicated per 5 min time slice. For the bar graph, statistical analysis was done by one-way ANOVA with Dunnett's test to assess the samples vs PTZ-only controls, with *P* values of <0.05 (*), <0.01 (**), and <0.001 (***).

the $GABA_A$ antagonist pentylenetetrazole (PTZ) in rodents[9] and in zebrafish (*Danio rerio*).[10,11]

In recent years, zebrafish have emerged as a novel experimental model for epilepsy.[10] PTZ, a proconvulsant widely used to induce absence seizures and generalized tonic-clonic seizures in rodents, induces a dose-dependent series of stereotypical behaviors in 7 day old zebrafish larvae, including clonus-type convulsions resembling epileptic seizures in mammals.[12] Epileptiform discharges were observed from electrophysiological recordings from the brains of PTZ-treated zebrafish larvae, with these discharges reduced by exposure to known anticonvulsant drugs such as valproate and diazepam. In order to facilitate the discovery of novel antiepileptic drugs, an automated tracking system was developed to monitor the movement of zebrafish larvae in 96-well plates.[11,13−15] Using this system, we found that similar antiepileptic drugs were found to suppress PTZ-induced seizurelike behaviors and electrographic activity in both zebrafish and mice, thereby validating the suitability of zebrafish for the high-throughput screening of potential anticonvulsant compounds.[11]

The general paradigm of using zebrafish as a primary model organism for high-throughput screening in the context of natural-products drug discovery has been gaining ground in the past decade.[16,17] The advantages of zebrafish as a biological vertebrate model (e.g., fecundity, rapid ex utero development, ease of husbandry, genetic similarities to rodents and humans), when coupled with robust chromatographic and spectroscopic methods, have contributed to discoveries and further development of hit compounds from various plant extracts.[13,15,18] The sharpening focus on exploring plant extracts as sources for novel anticonvulsant drug candidates is not without compelling pretext, such as the historical use of medicinal plants to treat epilepsy,[19,20] supported by anticonvulsant natural-product molecules isolated from such plants to date.[13,15] The impetus for such efforts can be attributed to the need to address drug-resistant epilepsy and to develop improved AEDs or even botanical therapeutics with reduced neurological and toxicological side effects,[20] especially since the potential of medicinal plants as source of chemical diversity for modern AED discovery cannot be disputed.

2. RESULTS AND DISCUSSION

Zebrafish-Based Screen of Southeast Asian Medicinal Plant Extracts. Prescreening was performed on nine Southeast Asian plants from the University of Strathclyde's natural product library (data not shown), which were selected on the basis of recorded ethnopharmacological use in Philippine pharmacopeia.[21] *Solanum torvum* was chosen for more rigorous assessment. *Solanum torvum* L. (Solanaceae) is a plant used in traditional medicine in South China and Southeast Asia as a sedative, digestive, hemostatic, and diuretic.[22] In the Philippines, this plant is traditionally used as an antiepileptic and antispasmodic.[21] The major constituents of this plant are steroidal saponins, which exhibit various bioactivities including antiviral, cytotoxic, antimicrobial, and anti-inflammatory.[23−26] Anticonvulsant activity has previously been reported for solasodine, a steroidal alkaloid isolated from another *Solanum*

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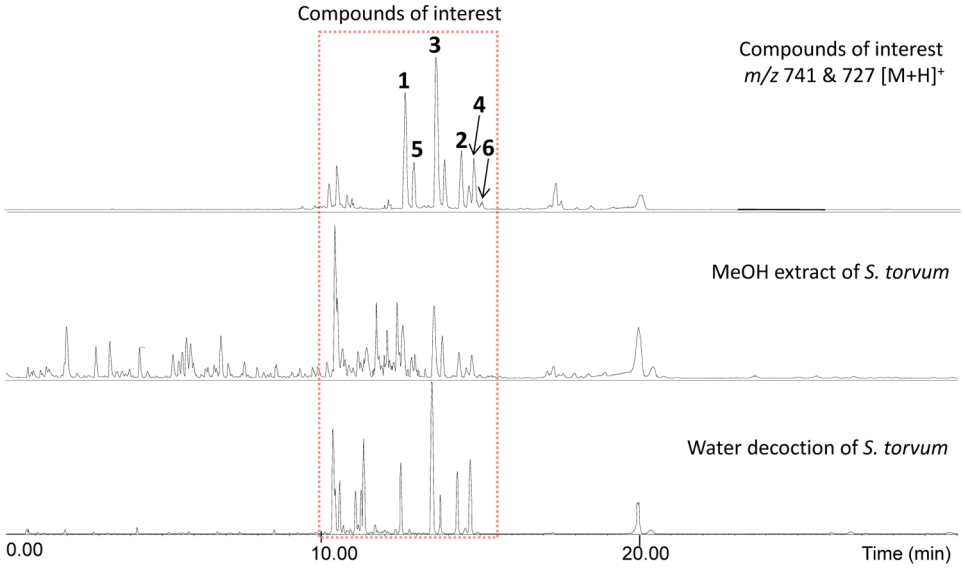

Figure 2. UHPLC-TOF-MS analysis of the methanolic extract and water decoction of *S. torvum*.

species (*S. sisymbriifolium*).[27] Based on these findings, *S. torvum* was chosen for further bioactivity analysis, metabolite profiling, and bioassay-guided microfractionation to identify its anticonvulsant constituents.

Bioactivity Analysis of *Solanum torvum*. The aerial parts of *S. torvum* were successively extracted with solvents of increasing polarity (hexane, dichloromethane, methanol, and water) and concentrated under vacuum. The plant was also extracted by water decoction according to the traditional preparation. Zebrafish larvae (at 6.5 dpf) were exposed to different concentrations of *S. torvum* crude extracts for 18 h before subsequent addition of PTZ, which induced seizurelike movement, such as swimming with increasing agitation leading to "whirlpool" behavior, loss of posture, and clonus-type movement. The methanolic and water extracts that were likely to be close in composition to the traditional usage of the plant exhibited significant reduction (35−40%) of PTZ-induced movement in 6/7 dpf larvae at 50 μg/mL ($p < 0.05$), with their maximum tolerated concentrations (MTCs) well beyond the highest tested concentration (200 μg/mL) (Figure 1).

Metabolite Profiling. The water decoction and methanolic extract were submitted to metabolite profiling by UHPLC-TOF-MS, and their composition was compared (Figure 2).[28] Based on the high resolution (HR)TOF-MS data obtained by metabolite profiling, the molecular formula of the compounds detected in the water decoction could be retrieved. These dereplication data revealed the presence of characteristic steroid glycosides isomers with a high molecular weight as the main LC peaks in the methanolic extract which were previously isolated from this plant.[23−26] Because of the presence of many isomers known to have various absolute configurations or glycoside substitution patterns, a full structure assignment could not be performed based on such data alone. The methanol extract was selected for further chemical study, as it exhibited reduction in PTZ-induced activity in zebrafish larvae at a comparable level to the aqueous extract, and presents a wider array of metabolites.

Bioactivity-Guided Microfractionation. In order to rapidly localize the chromatographic zones of the HPLC chromatogram holding the anticonvulsant activity, reversed phase semipreparative HPLC microfractionation of the crude methanolic extract (122 mg) was performed for metabolite profiling affording eight fractions (F1−F8) (Figure 3A).[29] Each microfraction was submitted to the zebrafish anticonvulsant bioassay. F6 and F7 showed a significant reduction of the epileptic effects of PTZ at 50 μg/mL (Figure 3B). The UHPLC-TOF-MS analysis of these latter microfractions showed mostly the presence of LC peaks having either a protonated molecules $[M + H]^+$ at m/z 741 or 727. This corresponded to isomeric compounds of molecular formula $C_{39}H_{64}O_{13}$ or $C_{38}H_{62}O_{13}$. Such data matched well with previously isolated steroid glycosides from this plant.[23−26] A differentiation of the various isomers present could however not be made based on MS data only. However, this rapid fractionation indicated that the steroid glycosides were responsible for the anticonvulsant activity of the extract measure in the in vivo assays.

In order to fully characterize the active compounds and assess their activities quantitatively, their isolation at a larger scale was needed. The separation was optimized by HPLC-UV-ELSD-MS. This procedure capitalizes on the HPLC modeling based on generic linear gradients at the analytical level to maximize the separation of interest compounds. These optimized analytical HPLC conditions were then transferred to medium pressure liquid chromatography (MPLC-UV-ELSD) using the same C_{18} reverse stationary phase (Zeoprep) and the same particle size (15−25 μm). Using this approach, six compounds were isolated in one step at the milligram scale (Figure 4).

Structural Elucidation of the Bioactive Compounds. Four compounds (**1**−**4**) displayed the same molecular formula ($C_{39}H_{64}O_{13}$) assigned from their ESI-HRMS positive ion at m/z 741.4446 (e.g., for Torvoside J) (calcd. for $C_{39}H_{65}O_{13}$ $[M + H]^+$: 741.4425). According to the Dictionary of Natural Products (The Chapman and Hall Dictionary of Natural Products on DVD, CRC Chemical Database), seven compounds, with the same spirostane skeleton, were found in the genus *Solanum* with this molecular formula. Namely, Torvoside C (this one was revised to Torvoside K by Iida et al.[30]), Torvoside J−L, Paniculonin B, Spirotorvoside, and Melongoside E. The ^{1}H NMR spectra of **1**−**4** showed a very similar pattern in agreement with glycosylated spirostane: two methyl singlets (Me-18 and Me-19), four methyl doublets, two

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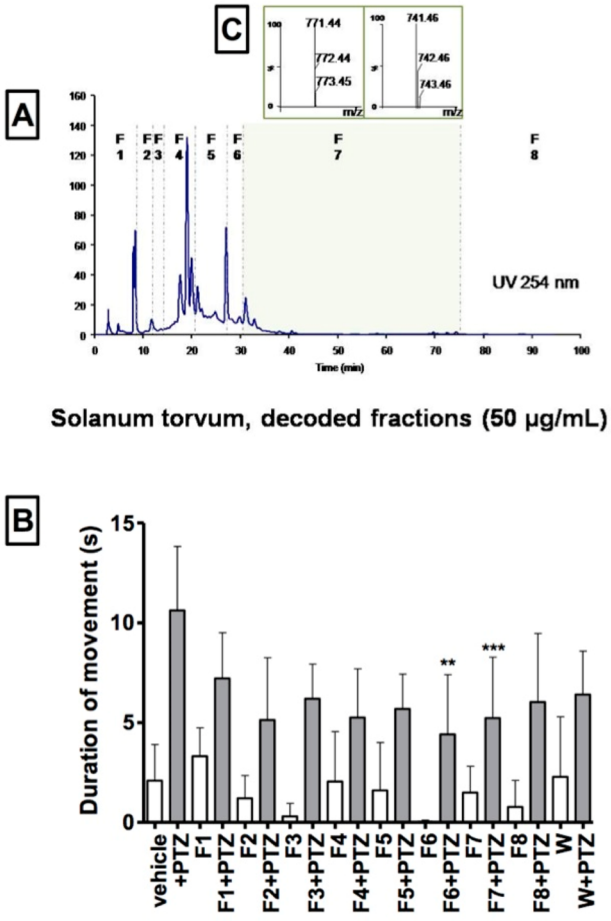

Figure 3. (A) HPLC microfractionation of the methanolic extract of *S. torvum*. (B) Total movement after 1 min manual tracking period (expressed in "duration of movement" in seconds) of major fractions of the plant extract, with statistical analysis done by one-way ANOVA with Dunnett's test to assess the samples vs PTZ-only controls, with *P* values of <0.05 (*), <0.01 (**), and <0.001 (***). (C) MS spectra of the main compounds present in the active zone.

belonging to the spirostane (Me-21 and Me-27) and two to the sugars moieties (Me-6′ and Me-6″), and two anomeric doublets at δ_H 5.14 (J = 1.7 Hz, H-1′) and 4.27 (J = 7.9 Hz, H-1″). The COSY, TOCSY, and HSQC spectra allowed identification of the sugars as a quinovose and a rhamnose. The long-range HMBC correlations from H-3′ of the quinovose (δ_H 3.42, t, J = 8.9 Hz) to C-1″ of the rhamnose (δ_C 102.8) and from H-1′ of the quinovose to C-6 of the spirostane (δ_C 80.4) indicated that the four compounds belong to the following type: 3*β*,6*α*,23-trihydroxy-5*α*-spirostane-6-*O*-*β*-D-rhamnopyranosyl-(1→3)-*O*-*β*-D-quinovopyranosyl. Melongoside E and Spirotorvoside being spirostane 3-*O*-glycoside, they were thus excluded from the list of possible structures. At this stage, Torvoside J−L and Paniculonon B remain as the four possible structures which differ only from their configuration at C-22, C-23, and C-25. According to Iida et al.,[30] the δ_C at C-20 and δ_H at Me-21 (in pyridine-d_5) are diagnostic for the determination of the configuration at C-22: δ_H 1.07−1.26 and δ_C 35.0−36.2 for 22-*α*-*O*-spirostanol (22-S) and δ_H 1.53−1.54 and δ_C43.1−44.1 for 22-*β*-*O*-spirostanol (22-R). As the NMR data have been recorded in CD_3OD (the solvent used for the dereplication database), a direct comparison with the reported data was not possible. However, compounds **1**−**4** could be split into two groups based on their chemical shift values at C-20 and Me-21. The ^{13}C chemical shifts recorded in CD_3OD being very close to those obtained in pyridine-d_5, we concluded that **1**−**3** were 22-R with the following δ_H(Me-21)/δ_C(C-20): 1.10/41.6, 1.12/40.8, and 1.16/43.6, whereas **4** exhibited a 22-S configuration (δ_H(Me-21)/δ_C(C-20): 0.96/37.0). The configuration of C-23 could be deduced from the H-23 coupling constant (dd (J = 10.3/5.2 Hz) when H-23 was in axial position and a triplet (J = 3 Hz) when H-23 was in equatorial position). The configuration at C-25 was usually determined by the signal pattern of H-26 (brd (J = 11.0 Hz) and dd (J = 11.0, 3.0 Hz) when H-25 is equatorial and t (J = 11.0 Hz) and brd (J = 11.0 Hz) when H-25 is axial). The δ_H of Me-27 was also characteristic as it appeared shifted downfield in axial position (1.10−1.16) compared to the equatorial position (0.77−0.82). Based on these observations, compound **1** was unambiguously identified as Torvoside J (22*R*,23*S*,25*S*), **2** as Torvoside L (22*R*,23*R*,25*S*), **3** as Torvoside K (22*R*,23*S*,25*R*)[30] and **4** to Paniculonin B (22S, 23S, 25S).[31]

The other isolated compounds (**5** and **6**) shared a same molecular $[M + H]^+$ ion at (eg *m/z 727.4302* for Paniculonin A) (calcd. for $C_{38}H_{63}O_{13}$ $[M + H]^+$: 727.4269) indicating a molecular formulas of $C_{38}H_{62}O_{13}$ shifted by 14 Da compare to **1**−**4**. The ^1H NMR spectrum of **5** was very close to that of Torvoside J (**1**) at the exception of the sugar region. The missing methyl doublet at δ_H 1.25 (CH_3-6″) in **1** and the lack of CH_2 in the molecular formulas suggested the presence of a pentose instead of a hexose. The second sugar was identified as a xylose (δ_H/δ_C 4.48/106.0 for H/C-1″, 3.26/75.3 for H/C-2″, 3.33/77.7 for H/C-3″, 3.49/71.0 for H/C-4″ and 3.23,3.90/67.1 for H/C-5″). Compound **5** was thus (22*R*,23*S*,25*S*)-3*β*,6*α*,23-trihydroxy-5*α*-spirostane 6-*O*-*β*-D-xylopyranosyl-(1→3)-*O*-*β*-D-quinovopyranoside.[32] The ^1H NMR of **6** displayed the same sugar pattern as **5** and the same spirostane pattern as Paniculonin B (**4**). Compound **6** was elucidated as Paniculonin A.[33] The NMR description of all these compounds have not yet been reported in CD_3OD, which is a common solvent used for at-line dereplication by micro-NMR methods, and they are thus detailed in the Methods.

Acid Hydrolysis of the Glycosides. Triterpene glycosides are known to have poor intestinal absorption mainly due to their unfavorable physicochemical properties, such as large molecular mass (>500 Da), high hydrogen-bonding capacity (>12), and high molecular flexibility (>10), that underlie poor membrane permeability.[34] Assuming that the isolated steroid glycosides from *S. torvum* may be pro-drugs, the fraction containing the triterpene glycosides was submitted to a mild acid hydrolysis (refluxed with 2% H_2SO_4/MeOH for 8 days at 37 °C) to provide the corresponding aglycones[35] that could mimic an initial degradation step in the stomach.[34] The hydrolysis afforded mainly one compound ($C_{22}H_{34}O_4$) ($[M + H]^+$ at *m/z* 363.2543 (calculated for $C_{22}H_{34}O_4$, 363.2535, 2.2 Δppm)). The NMR analysis of the HSQC spectrum of the hydrolyzed product revealed the absence of sugar signals as well as the characteristic signals belonging to the spirostane ring (e.g., the oxymethine CH-23 and oxymethylene CH_2-26). Moreover, a careful analysis of signals indicated that the hydrolyzed product consist of a 65/35 mixture of two aglycones as seen by the duplication of signals for H-16 (δ_H/δ_C 5.03/84.6 and 4.83/84.1), H-20 (δ_H/δ_C 2.60/37.4 and 2.97/38.7), H-17 (δ_H/δ_C 1.98/59.9 and 22.31/56.6), Me-21 (δ_H/δ_C 1.28/17.9 and 1.30/10.3), and Me-18 (δ_H/δ_C 0.73/13.9 and 0.81/14.0). Despite these differences in chemical shifts, the HMBC

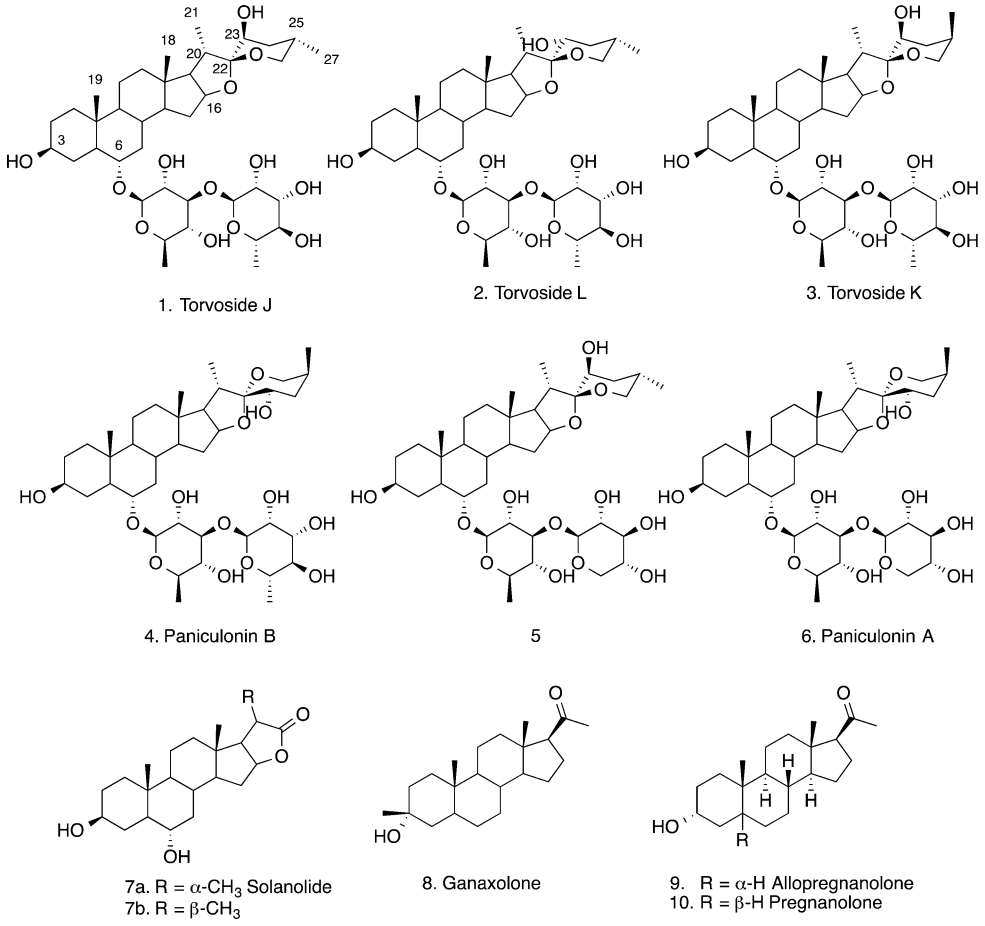

Figure 4. Isolated compounds from the methanolic extract of *S. torvum*, the common aglycone obtained by acid hydrolysis of the extract, and the structure of the ganaxolone, allopregnanolone, and pregnanolone. Compound **5**: (22*R*,23*S*,25*S*)-3*β*,6*α*,23-trihydroxy-5*α*-spirostane-6-*O*-*β*-D-xylopyranosyl-(1→3)-*O*-*β*-D-quinovopyranoside.

correlations were the same in both molecules: from Me-21 to C-17, C-20, and the ester carbonyl at δ_C 183.9/182.2 and from Me-18 to C-12, C-13, C-14, and C-17. The two aglycones were thus epimers. Analysis of the NOESY correlations indicated that the first one was solanolide[36] as seen by the dipolar correlation from H-16 to Me-21 and H-17 and from H-20 and Me-18. For the second isomer, the NOE crosspeaks from H-16 to H-17 and H-20 and from Me-21 to Me-18 showed that it was the epimer of solanolide at C-20. The mechanism for production of these aglycones (**7a** and **7b**) was already speculated for the study of similar compounds in *Solanum anguivi*.[37]

Bioactivity Analysis of Isolated Compounds in Vivo. The six isolated *S. torvum* steroidal glycosides and the aglycone from acid hydrolysis (compounds **1**−**7**) were subjected to further biological activity analyses. The selected compounds were exposed to 6/7 dpf zebrafish larvae for 18 h in various concentrations, with their MTC values beyond the highest concentration (280 μM, with the exception of compound **1**, which was at 140 μM), before subsequent exposure to 20 mM PTZ (Figure 5). Compounds **4**−**6** did not show significant reduction in PTZ-induced locomotor activity in larvae (Figure 5-4, -5, and -6). Inconsistent reduction of PTZ-induced activity over the 30 min exposure time was seen for compounds **2** and **3** (Figure 5-2 and -3), with compound **3** possibly saturating the receptors involved with PTZ-induced movement at all three concentrations (Figure 5-3), although they display reduction of total movement only at 70 μM (Figure 5-7). Compound **1** showed significant reduction of PTZ-induced activity from 70 μM (moderate, $P < 0.05$) until 140 μM ($P < 0.001$) (Figure 5-1). As these compounds have similar structures, the slight difference in compound **1** (the stereochemical positions of −OH and $-CH_3$ in the spirostane ring) compared to the other identified torvosides (**2**, **3**) may play a role in activity modulation, as well as the type of sugar moiety attached to the aglycone (**5**) (Figure 4). However, significant reduction of PTZ-induced activity in larvae was also observed for acid-hydrolysis aglycone mix (**7a** and **b**) at its MTC value (140 μM) (Figure 6A), indicating that the presence of a sugar moiety may not be necessary.

The structures of the steroidal glycosides isolated from *S. torvum*, especially their acid hydrolysis aglycone product, bear resemblance to neurosteroids (e.g., alphaxalone, pregnanolone, allopregnanolone, ganaxolone) which are known to interact with GABA receptors and have been used as anesthetics and sedatives.[38,39] One such neurosteroid, ganaxolone, a potent $GABA_A$ modulator, shows anticonvulsant properties[40] and is now facing phase III clinical trials on adult patients suffering from partial seizures.[41] Based on the similarity of structure to the isolated compounds and its known pharmacology and mode of action, ganaxolone was selected as a reference drug for comparison purposes in larval zebrafish PTZ assays. Upon exposure to larval zebrafish prior to addition of PTZ, ganaxolone (**8**) has shown to significantly reduce the influence

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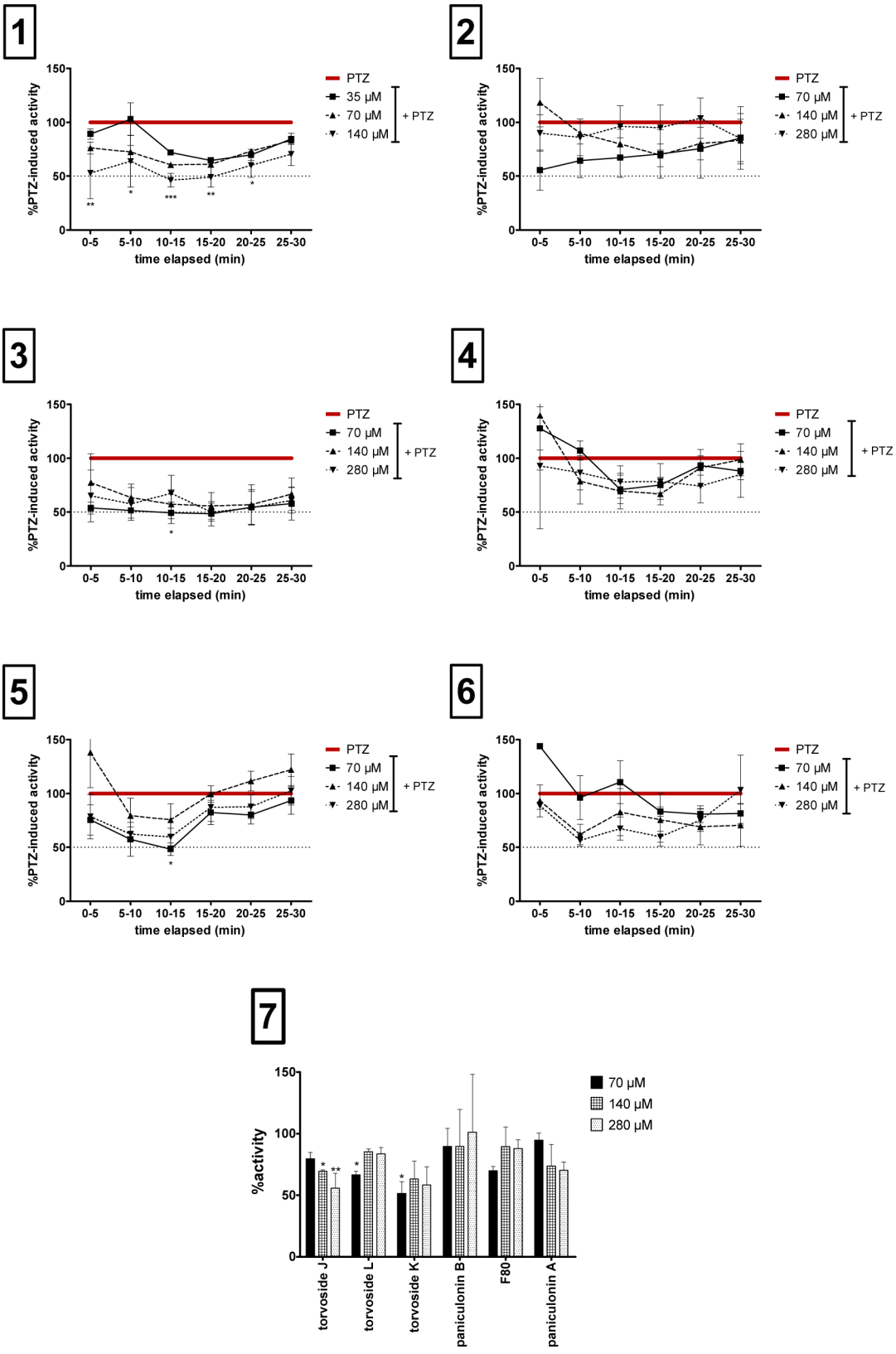

Figure 5. PTZ-induced activity curve for a 30 min tracking period of 7 dpf zebrafish larvae after 18 h pretreatment with different concentrations of compounds **1**–**6** (number labels on each graph correspond to compound). Total movement after 30 min tracking period (expressed in %activity) represented in a bar graph (7) below. All results were normalized against PTZ controls (set at 100%). Analysis of the activity curves was done by two-way ANOVA, with *P* values of <0.05 (*), <0.01 (**), and <0.001 (***) indicated per time period. For the bar graph, statistical analysis was done by one-way ANOVA with Dunnett's test to assess the samples vs PTZ-only controls, with *P* values of <0.05 (*), <0.01 (**), and <0.001 (***).

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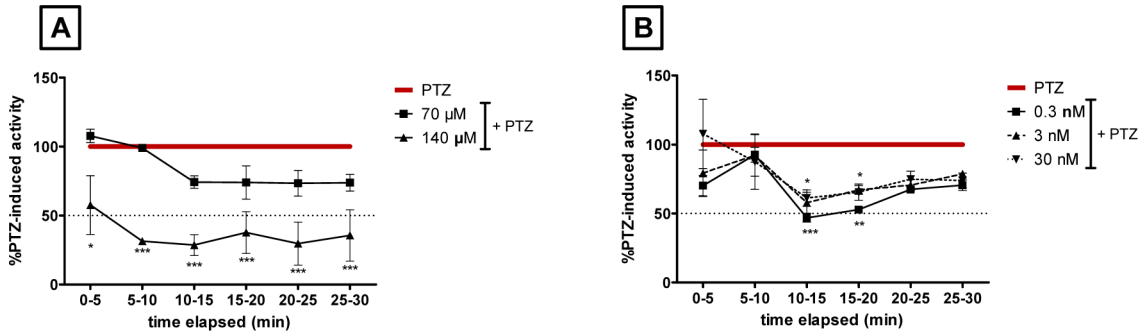

Figure 6. PTZ-induced activity curve for a 30 min tracking period of 7 dpf zebrafish larvae after 18 h pretreatment with different concentrations of (A) compound **7** (solanolide) and (B) compound **8** (ganaxolone). All results were normalized against PTZ controls (set at 100%). Analysis was done by two-way ANOVA, with *P* values of <0.05 (*), <0.01 (**), and <0.001 (***) indicated per time period.

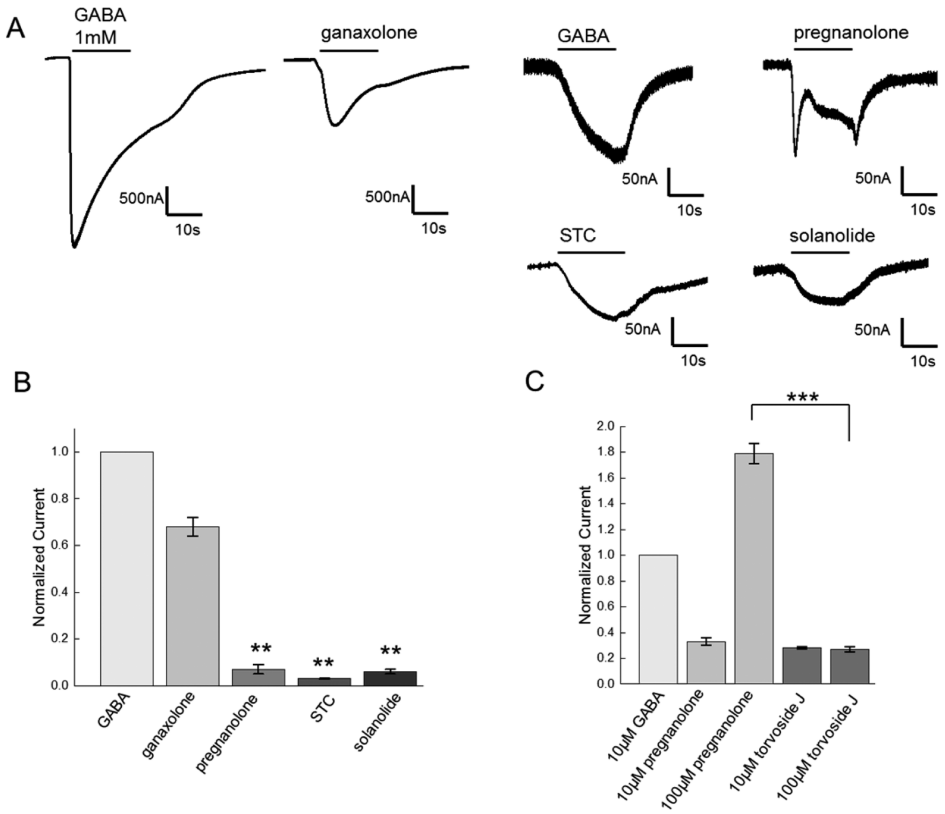

Figure 7. (A) Representative current traces generated in response to 1 mM GABA or 10 μM of indicated compounds and 200 μg/mL of *S. torvum* crude (STC) extract applied for 15 s on *Xenopus laevis* oocytes expressing $\alpha1\beta2\gamma2$s receptors. (B) Responses to application of indicated compounds normalized to 1 mM GABA applied at the beginning of each recording. Compared to ganaxolone, significant reduction in the current amplitude with $P < 0.01$ (**), determined using one way ANOVA with Dunnett's test, was found for all tested compounds; $n = 4–6$. (C) Responses to compounds applied together with 10 μM GABA in 10 or 100 μM concentrations and normalized to the response to 10 μM GABA applied at the beginning of recording. Washing time between applications was 5 min. Statistical analysis was done by repeated measures ANOVA with Bonferroni correction comparing applications in the corresponding concentrations, $P < 0.001$ (***); $n = 4$.

of PTZ on the locomotor activity of 7 dpf zebrafish larvae at 0.3 nM (Figure 6B). Comparing the reduction of PTZ-induced activity of ganaxolone with any of the isolated *S. torvum* compounds (and aglycone), we observed that there is a large difference in the order of magnitude in terms of potencies of the compounds.

Bioactivity Analysis of Isolated Compounds in Vitro. Since neurosteroids are known to be $GABA_A$ modulators and the aglycone (**7**) present a related steroid structural type, we used the *Xenopus laevis* oocyte expression system to test the effects of different compounds on the activity of $GABA_A$ receptors comprising $\alpha1$, $\beta2$, and $\gamma2$s subunits.[42] To check the expression level, a test pulse with 1 mM GABA was applied at the beginning of each recording. After thorough washing, 10 μM ganaxolone, pregnanolone, and the aglycone (**7**) or 200 μg/mL *S. torvum* crude extract were applied and the obtained response normalized to the test pulse (Figure 7A, B). Pregnanolone, the crude extract and the aglycone (**7**) produced significantly smaller currents compared to the currents generated by ganaxolone (**8**), $P < 0.01$, $n = 4–6$. All isolated steroid glycosides elicited currents resembling the ones induced by 10 μM GABA (Figure 7A).

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In a different set of experiments (Figure 7C), we examined the potentiation effect on $GABA_A$ receptor response known for pregnanolone and allopregnanolone.[43] Here the first application of 10 μM GABA was followed by application of 10 μM GABA together with 10 or 100 μM of each tested compound. Washing times in between applications was 5 min, and all responses were normalized to the current produced by 10 μM GABA in the same cell. Except for application of 10 μM GABA together 100 μM pregnanolone, all other currents were 2−3 fold reduced compared to the ones generated by 10 μM GABA (Figure 7C). These results indicate that the isolated compounds from *S. torvum* are only weak modulators of GABA receptors compared to ganaxolone or pregnanolone.

The 4- to 6-fold difference in normalized current response of the isolated glycosides and aglycone, versus ganaxolone, in the *X. laevis* oocyte system appears to correlate well with the difference in potencies in the larval zebrafish assay. The disparate order of magnitude of difference in potencies (subnanomolar vs micromolar) between ganaxolone and the *S. torvum* aglycone (or even the parent steroidal glycosides) in the larval zebrafish assay may be accounted for the structural differences between them. The fundamental criterion for a neurosteroid to be effective in modulating GABA receptors is that the compound should be at least a 3α-OH steroid.[44,45] Although being of the same structural type as ganaxolone, the acid-hydrolyzed aglycone (7) is a 3β-OH steroid rather than its alpha equivalent.

These results indicate that the steriodal glycosides of *S. torvum* are significantly less potent as $GABA_A$ modulators in comparison to ganaxolone, as evidenced both by their activity in the zebrafish PTZ-induced seizure model as well as on $GABA_A$ receptors expressed in *X. laevis* oocytes. It is therefore possible that the in vivo anticonvulsant activity of these steriodal glycosides may also involve mechanisms other than $GABA_A$ modulation.

3. CONCLUSION

This work presents a new strategy of combining HPLC microfractionation with at-line bioactivity assessment on an in vivo zebrafish behavioral epilepsy assay. It has allowed an efficient localization of bioactive steroid glycosides and has enabled the efficient targeted isolation of the bioactive compounds.

The isolation procedure was performed by direct geometrical gradient transfer from analytical HPLC-ELSD to preparative medium pressure liquid chromatography (MPLC-ELSD) and allowed an efficient isolation of the active compounds in large amounts. The bioguided isolation afforded six spirostane glycosylated triterpenes that were responsible for the antiseizure activity of the decoction and methanolic extract of aerial parts of *Solanum torvum*. These compounds presented anticonvulsant activity in the in vivo zebrafish model but no specificity was recorded among the different glycosides tested. Since the presumed bioavailability of such compounds is poor and they might undergo hydrolysis when taken orally, as it the case with the traditional preparation, we demonstrated that these compounds could generate an active aglycone through a skeleton rearrangement under mild hydrolysis conditions. Despite the structural resemblance to neurosteroids such as ganaxolone, these isolated steroidal glycosides and aglycone exhibit only weak GABA modulation, suggesting that these compounds may act via a different mechanism to significantly reduce PTZ-induced seizures in zebrafish.

4. METHODS

General Experimental Procedures. NMR spectroscopic data were recorded on a 500 MHz Agilent/Varian Inova spectrometer. Chemical shifts are reported in parts per million (δ) using the residual CD_3OD signal (δ_H 3.31; δ_C 49.0) as internal standards for ^1H and ^{13}C NMR, and coupling constants (J) are reported in Hz. Complete assignment was performed based on 2D experiments (COSY, TOCSY, NOESY, edited-HSQC, and HMBC). ESI-HRMS data were obtained on a Micromass LCT Premier time-of-flight mass spectrometer from Waters with an electrospray ionization (ESI) interface (Waters, Milford, MA). Analytical HPLC was performed using an HP 1100 system equipped with a photodiode array detector (Agilent Technologies, Santa Clara, CA). MPLC was performed using a Büchi 681 pump (Büchi, Flawil, Switzerland) equipped with a Knauer UV detector (Knauer, Berlin, Germany) and a 920 × 49 mm i.d. column (Büchi, Flawil, Switzerland) loaded with ZEOprep C18 as the stationary phase 15−25 μm (Zeochem, Uetikon am See, Switzerland).

Plant Material. The aerial parts of *Solanum torvum* were collected in Infanta, Quezon in the Philippines in 2011. The botanical material was properly identified, with voucher specimen #8790, deposited in the Herbarium of the Institute of Biology of the University of the Philippines (UP Diliman). A Material Transfer Agreement for the collection and transfer of *S. torvum* material from the Philippines to Belgium, in concordance with the UN Convention on Biodiversity, has been signed between KU Leuven and Ateneo de Manila University.

Preparation of Extract. The dried aerial parts (102.24 g) were successively extracted under maceration and agitation with increasing polarity extraction in a round-bottom flask using a sequence of solvents of increasing polarity (hexane, dichloromethane, methanol, and water) and concentrated under vacuum to yield 2.15 g of hexane (2.1%), 1.73 g of dichloromethane (1.7%), and 13.59 g of methanol (13.3%). The aqueous extract was frozen and lyophilized, yielding 1.83 g (1.8%) of water extract. A traditional decoction was prepared using 10 g of dried aerial part of *S. torvum* in 100 mL of hot water for 1 h. The solution was filtered and evaporated to dryness (1.6 g [16%]).

HPLC-DAD-ELSD-MS Analysis. HPLC-DAD-ELSD-MS data were obtained with an Agilent HP 1100 series system consisting of an autosampler, high-pressure mixing pump, and DAD detector (Agilent Technologies, Santa Clara, CA) connected to a Finnigan MAT LCQ ion trap mass spectrometer (Finnigan, San Jose, CA) equipped with a Finnigan electrospray interface (ESI) and a ELSD detector Sedex 85 (Sedere, Oliver, France). The HPLC conditions were as follows: a Zeoprep C18 column (15−25 μm, 250 × 4.6 mm i.d.; this particle size was selected for an optimum gradient transfert the MPLC) (Zeochem, Uetikon am See, Switzerland); solvent system: A, H_2O containing 0.1% formic acid and B, ACN containing 0.1% formic acid; step gradient: 2−27% of B in 5 min followed by 27% of B for 20 min, then 27% B to 36% of B in 5 min, followed by 36% B for 20 min and 36% to 95% of B in 10 min; flow rate: 1 mL min^{-1}; injection volume: 10 μL; and sample concentration: 10 mg/mL in MeOH. The samples were analyzed with UV detection, and the absorbance was measured at 280 nm and ELSD detection. ESI-MS conditions were as follows: capillary voltage: 30 V; capillary temperature: 200 °C; source voltage: 4.5 kV; source current: 80 μA; nitrogen as the sheath gas; and positive and negative ion mode. Spectra (180−1200 mu) were recorded every 3 s.

UHPLC-TOF-HRMS Analyses. HRMS metabolite fingerprint of the extracts was performed on a Micromass-LCT Premier Time of Flight (TOF) mass spectrometer (Waters, Milford MA) equipped with an electrospray interface and coupled to an Acquity UPLC system (Waters, Milford MA). The ESI conditions were as follows: capillary voltage: 2800 V;, cone voltage: 40 V; MCP detector voltage: 2400 V; source temperature: 120 °C; desolvation temperature: 300 °C; cone gas flow: 20 L/h; and desolvation gas flow: 600 L/h. Detection was performed in positive ion mode (PI) with a m/z range of 100−1300 Da and a scan time of 0.5 s in the W-mode. The MS was calibrated using sodium formate, and leucine encephalin (Sigma-Aldrich, Steinheim, Germany) was used as an internal reference at 2 μg/mL

and infused through a Lock Spray probe at a flow rate of 10 μL/min with the help of a second LC pump.

The general profiling of the extract was performed on a Acquity BEH C18 UPLC column (150 × 2.1 mm i.d.; 1.7 μm, Waters, Milford, MA) using a linear gradient from 5% ACN + 0.1% FA to 95% of B in 30 min, while for the separation of the isomers the following otpimised conditions were used: Acquity BEH C18 UPLC column (50 × 2.1 mm i.d.; 1.7 μm, Waters, Milford, MA) using a stepgradient (solvent system: A = 0.1% formic acid−water, B = 0.1% formic acid−acetonitrile; gradient: 5−36% B in 1 min, then 36% B to 36% B in 2 min, 36% B to 95% B in 1.5 min; flow rate 1.1 mL/min). The temperature was set to 40 °C. The injected volume was kept constant (1 μL).

Semipreparative HPLC-UV Microfractionation. A first fractionation allowed the localization of the active area on the chromatogram. The fractionation was performed on a5 μm Xterra prep Column C18 OBD (150 × 19 mm, 5 μm). The gradient started with 100% of mobile phase A (H_2O + 0.1% FA) to 60% of mobile phase B (ACN + 0.1% FA) in 56 min.[13] The fractionation was monitored by UV, with fractions collected every 3 min. Fraction 1 has been obtained by pooling three successives fractions, F4 and F5 by pooling two successives fractions. The non-UV-active part was pooled into two main fractions (F8 and F9).

Isolation of Active Compounds from the Methanolic Extract. The methanolic extract (4 g) was first fractionated using MPLC with Zeoprep C18 as the stationary phase (15−25 μm, 920 × 49 mm i.d.) (Zeochem, Uetikon am See, Switzerland) ACN and H_2O containing 0.1% formic acid as mobile phase in a step gradient as the following: 2−27% B for 9.5 h, isocratic 27% B for 31.5 h, 27−36% B for 8 h, isocratic 36% for 32 h, 36−95% B for 16 h, isocratic 95% B for 16 h for 113 h as a total of purification time. The flow rate was 4 mL min^{-1}, and the UV absorbance was detected at 280 nm. The ELSD detection was performed under the following parameters: pressure 3.2 bar, 45 °C, split to provide a 500 μL/min flow rate, gain 8. The MPLC yielded 182 fractions. All fractions were analyzed by UHPLC-TOF-MS. Fraction 71 yielded compound **1**, torvoside J (9.4 mg), fraction 80 yielded **2**, F80 (3.4 mg), fraction 88A yielded **3**, torvoside K (7 mg), fraction 88B yielded **4**, torvoside L (7 mg), fraction 117 yielded **5**, paniculonin B (2.2 mg), and fraction 128 yielded **6**, paniculonin A (1.6 mg).

Hydrolysis Conditions for Obtaining the Aglycone 7. In order to obtain the aglycone from the spirostane glycoside, a mild hydrolysis has been performed. Indeed, classical acidic hydrolysis induces a destruction of the spirostane. The mild hydrolysis protocol was based on the one described by Kesselmeier et al.,[35] in 1979, except the HCl has been replaced by H_2SO_4. Aglycone was obtained by subjecting the glycoside of interest to a solution of 2% H_2SO_4 in MeOH at 37 °C during 8 days.

Spectral Data of the Isolated Compounds. *Compound 1, Torvoside J.* ^{1}H NMR (CD_3OD, 500 MHz) δ 0.68 (1H, td, *J* = 11.3, 3.6 Hz, H-9), 0.77 (3H, d, *J* = 6.6 Hz, Me-27), 0.81 (3H, s, Me-18), 0.87 (3H, s, Me-19), 0.95 (1H, m, H-7b), 1.02 (1H, m, H-1b), 1.10 (3H, d, *J* = 6.9 Hz, Me-21), 1.13 (1H, m, H-12b), 1.15 (2H, m, H-4b, H-5), 1.16 (1H, m, H-14), 1.25 (3H, d, *J* = 6.2 Hz, Me-6″), 1.27 (1H, m, H-15b), 1.28 (3H, d, *J* = 6.2 Hz, Me-6′), 1.32 (1H, m, H-11b), 1.41 (1H, m, H-2b), 1.54 (1H, m, H-11a), 1.61 (1H, m, H-24b), 1.64 (1H, m, H-8), 1.66 (1H, m, H-24a), 1.69 (1H, m, H-17), 1.70 (1H, m, H-1a), 1.74 (1H, m, H-12a), 1.75 (1H, m, H-2a), 1.98 (1H, m, H-15a), 2.04 (1H, m, H-25), 2.17 (1H, dt, *J* = 12.5, 4.1 Hz, H-7a), 2.25 (1H, p, *J* = 6.9 Hz, H-20), 2.38 (1H, m, H-4a), 3.02 (1H, t, *J* = 8.9 Hz, H-4′), 3.28 (1H, dd, *J* = 8.9, 7.9 Hz, H-2′), 3.31 (1H, m, H-5′), 3.36 (1H, m, H-26b), 3.38 (1H, m, H-6), 3.40 (1H, t, *J* = 9.6 Hz, H-4″), 3.43 (1H, t, *J* = 8.9 Hz, H-3′), 3.46 (2H, m, H-3, H-26a), 3.53 (1H, t, *J* = 3.0 Hz, H-23), 3.70 (1H, dd, *J* = 9.6, 3.3 Hz, H-3″), 3.94 (1H, brs, H-2″), 3.99 (1H, dq, *J* = 9.6, 6.2 Hz, H-5″), 4.27 (1H, d, *J* = 7.9 Hz, H-1′), 4.46 (1H, q, *J* = 6.9 Hz, H-16), 5.14 (1H, brs, H-1″). ^{13}C NMR (CD_3OD, 126 MHz) δ 13.9 (C-19), 16.8 (C-18), 17.1 (C-21), 17.4 (C-27), 17.9 (C-6″), 18.4 (C-6′), 22.0 (C-11), 24.9 (C-25), 31.8 (C-2), 32.7 (C-4), 32.9 (C-15), 35.2 (C-8), 37.5 (C-10), 37.6 (C-24), 38.4 (C-1), 40.7 (C-12), 41.6 (C-7, C-20), 42.0 (C-13), 51.7 (C-5), 55.0 (C-9), 57.4 (C-14), 65.6 (C-17), 67.5 (C-26), 70.0 (C-5″), 71.0 (C-23), 71.8 (C-3), 72.2 (C-3″), 72.3 (C-2″), 72.9 (C-5′), 73.9 (C-4″), 75.7 (C-4′), 76.3 (C-2′), 80.3 (C-6), 82.3 (C-16), 84.2 (C-3′), 102.7 (C-1″), 105.1 (C-1′), 109.9 (C-22). ESI-HRMS *m/z* 741.4437 $[M + H]^+$ (calculated for $C_{39}H_{65}O_{13}$, 741.4425, Δppm = 1.6).

Compound 2, Torvoside L. ^{1}H NMR (CD_3OD, 500 MHz) δ 0.69 (1H, m, H-9), 0.82 (3H, d, *J* = 6.4 Hz, Me-27), 0.87 (3H, s, Me-18), 0.88 (3H, s, Me-19), 0.94 (1H, q, *J* = 12.2 Hz, H-7b), 1.02 (1H, brt, *J* = 12.7 Hz, H-1b), 1.12 (1H, m, H-14), 1.14 (1H, m, H-4b), 1.16 (2H, m, H-5, H-12b), 1.16 (3H, d, *J* = 7.2 Hz, Me-21), 1.24 (3H, d, *J* = 6.2 Hz, Me-6″), 1.28 (1H, m, H-15b), 1.28 (3H, d, *J* = 6.2 Hz, Me-6′), 1.35 (1H, m, H-11b), 1.41 (1H, m, H-2b), 1.48 (1H, q, *J* = 11.8 Hz, H-24b), 1.55 (1H, d, *J* = 13.7 Hz, H-11a), 1.66 (1H, m, H-8), 1.71 (1H, m, H-1a), 1.74 (1H, m, H-12a), 1.75 (2H, m, H-2a, H-25), 1.79 (1H, m, H-24a), 1.84 (1H, dd, *J* = 9.1, 6.3 Hz, H-17), 1.96 (1H, dq, *J* = 12.1, 6.5 Hz, H-15a), 2.17 (1H, m, H-7a), 2.20 (1H, m, H-20), 2.38 (1H, m, H-4a), 3.02 (1H, t, *J* = 9.1 Hz, H-4′), 3.26 (1H, dd, *J* = 9.3, 8.0 Hz, H-2′), 3.30, (1H, m, H-26b), 3.31 (1H, m, H-5′), 3.39 (2H, m, H-6, H-4″), 3.42 (1H, m, H-3′), 3.43 (1H, m, H-26a), 3.45 (1H, m, H-3), 3.63 (1H, dd, *J* = 11.5, 4.6 Hz, H-23), 3.69 (1H, dd, *J* = 9.5, 3.4 Hz, H-3″), 3.93 (1H, dd, *J* = 3.4, 1.8 Hz, H-2″), 3.99 (1H, m, H-5″), 4.27 (1H, d, *J* = 8.0 Hz, H-1′), 4.71 (1H, q, *J* = 7.6 Hz, H-16), 5.14 (1H, d, *J* = 1.8 Hz, H-1″). ^{13}C NMR (CD_3OD, 126 MHz) δ 13.6 (C-19), 16.2 (C-21), 16.6 (C-27), 17.0 (C-18), 17.7 (C-6″), 18.2 (C-6′), 21.8 (C-11), 31.6 (C-25), 31.8 (C-2), 32.5 (C-4), 34.5 (C-15), 34.9 (C-8), 38.2 (C-1), 38.3 (C-24), 41.0 (C-12), 41.5 (C-7), 43.6(C-20), 51.6 (C-5), 54.9 (C-9), 56.5 (C-14), 64.1 (C-17), 69.3 (C-26), 69.9 (C-5″), 70.6 (C-23), 71.7 (C-3), 72.1 (C-3″), 72.2 (C-2″), 72.8 (C-5′), 73.9 (C-4″), 75.5 (C-4′), 76.2 (C-2′), 80.2 (C-6), 84.2 (C-3′), 85.2 (C-16), 102.6 (C-1″), 104.9 (C-1′). ESI-HRMS *m/z* 741.4446 [M + H]+ (calculated for $C_{39}H_{65}O_{13}$, 741.4425, Δppm = 2.8).

Compound 3, Torvoside K. ^{1}H NMR (CD_3OD, 500 MHz) δ 0.69 (1H, m, H-9), 0.82 (3H, s, Me-18), 0.87 (3H, s, Me-19), 0.95 (1H, m, H-7b), 1.02 (1H, m, H-1b), 1.12 (3H, d, *J* = 7.0 Hz, Me-21), 1.14 (2H, m, H-4b, H-12b), 1.16 (1H, m, H-5), 1.17 (1H, m, H-14), 1.21 (3H, d, *J* = 7.3 Hz, Me-27), 1.24 (3H, d, *J* = 6.2 Hz, Me-6″), 1.27 (1H, m, H-15b), 1.28 (3H, d, *J* = 6.1 Hz, Me-6′), 1.32 (1H, m, H-11b), 1.40 (1H, m, H-2b), 1.52 (1H, m, H-24b), 1.55 (1H, m, H-11a), 1.65 (1H, m, H-8), 1.67 (1H, m, H-25), 1.70 (1H, m, H-17), 1.71 (1H, m, H-1a), 1.75 (2H, m, H-2a, H-12a), 1.96 (1H, m, H-15a), 2.06 (1H, m, H-24a), 2.17 (1H, m, H-7a), 2.35 (1H, m, H-20), 2.38 (1H, m, H-4a), 3.01 (1H, t, *J* = 9.0 Hz, H-4′), 3.26 (1H, dd, *J* = 9.0, 7.9 Hz, H-2′), 3.31 (2H, m, H-26b, H-5′), 3.39 (2H, m, H-6, H-4″), 3.42 (1H, t, *J* = 9.0 Hz, H-3′), 3.45 (1H, m, H-3), 3.57 (1H, t, *J* = 3.9 Hz, H-23), 3.69 (1H, dd, *J* = 9.5, 3.4 Hz, H-3″), 3.93 (1H, dd, *J* = 3.4, 1.7 Hz, H-2″), 3.96 (1H, dd, *J* = 11.2, 3.8 Hz, H-26a), 3.99 (1H, dq, *J* = 9.5, 6.2 Hz, H-5″), 4.27 (1H, d, *J* = 7.9 Hz, H-1′), 4.47 (1H, q, *J* = 7.8 Hz, H-16), 5.14 (1H, d, *J* = 1.7 Hz, H-1″). ^{13}C NMR (CD_3OD, 126 MHz) δ 13.6 (C-19), 16.6 (C-18), 16.7 (C-21), 17.7 (C-6″), 18.1 (C-6′), 20.0 (C-27), 21.9 (C-11), 27.7 (C-25), 31.6 (C-2), 32.4 (C-4), 32.7 (C-15), 34.3 (C-24), 35.0 (C-8), 38.3 (C-1), 40.7 (C-12), 40.8 (C-20), 41.5 (C-7), 51.6 (C-5), 54.9 (C-9), 57.3 (C-14), 65.2 (C-17), 66.0 (C-26), 69.8 (C-5″), 71.0 (C-23), 71.6 (C-3), 72.0 (C-3″), 72.2 (C-2″), 72.8 (C-5′), 73.9 (C-4″), 75.5 (C-4′), 76.2 (C-2′), 80.2 (C-6), 82.4 (C-16), 84.0 (C-3′), 102.7 (C-1″), 105.0 (C-1′). ESI-HRMS *m/z* 741.4453 [M + H]+ (calculated for $C_{39}H_{65}O_{13}$, 741.4425, Δppm = 3.8).

Compound 4, Paniculonin B. ^{1}H NMR (CD_3OD, 500 MHz) δ 0.69 (1H, td, *J* = 12.0, 3.9 Hz, H-9), 0.86 (3H, s, Me-18), 0.88 (3H, s, Me-19), 0.95 (1H, m, H-7b), 0.96 (3H, d, *J* = 7.0 Hz, Me-21), 1.03 (1H, m, H-1b), 1.11 (3H, d, *J* = 6.8 Hz, Me-27), 1.14 (1H, m, H-4b), 1.16 (1H, m, H-5), 1.17 (1H, m, H-12b), 1.18 (1H, m, H-14), 1.24 (3H, d, *J* = 6.2 Hz, Me-6″), 1.27 (3H, d, *J* = 6.1 Hz, Me-6′), 1.33 (1H, m, H-11b), 1.41 (2H, m, H-2b, H-15b), 1.55 (1H, brd, *J* = 13.6 Hz, H-11a), 1.67 (2H, m, H-8, H-24b), 1.71 (1H, m, H-1a), 1.75 (2H, m, H-2a, H-12a), 1.76 (1H, m, H-17), 1.89 (1H, m, H-25), 1.90 (1H, m, H-24a), 1.96 (1H, m, H-15a), 2.18 (1H, dt, *J* = 12.5, 4.0 Hz, H-7a), 2.38 (1H, m, H-4a), 2.53 (1H, p, *J* = 7.0 Hz, H-20), 3.01 (1H, t, *J* = 9.0 Hz, H-4′), 3.19 (1H, d, *J* = 11.0 Hz, H-26b), 3.26 (1H, dd, *J* = 9.0, 7.9 Hz, H-2′), 3.31 (1H, m, H-5′), 3.39 (1H, t, *J* = 9.5 Hz, H-4″), 3.39 (1H, m, H-6), 3.42 (1H, t, *J* = 9.0 Hz, H-3′), 3.45 (1H, m, H-3), 3.67 (1H, dd,

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J = 11.3, 5.0 Hz, H-23), 3.69 (1H, dd, J = 9.5, 3.3 Hz, H-3″), 3.85 (1H, dd, J = 11.0, 2.4 Hz, H-26a), 3.93 (1H, dd, J = 3.3, 1.8 Hz, H-2″), 3.99 (1H, dq, J = 9.5, 6.2 Hz, H-5″), 4.27 (1H, d, J = 7.9 Hz, H-1′), 4.44 (1H, q, J = 7.5 Hz, H-16), 5.14 (1H, d, J = 1.8 Hz, H-1″). ^{13}C NMR (CD_3OD, 126 MHz) δ 13.8 (C-19), 14.3 (C-21), 17.0 (C-18), 17.6 (C-27), 17.9 (C-6″), 18.4 (C-6′), 22.1 (C-11), 31.3 (C-25), 31.9 (C-2), 32.6 (C-15), 32.7 (C-4), 35.1 (C-8), 36.0 (C-24), 37.0 (C-20), 37.6 (C-10), 38.5 (C-1), 41.3 (C-12), 41.7 (C-7), 42.2 (C-13), 51.8 (C-5), 55.1 (C-9), 57.4 (C-14), 63.2 (C-17), 64.0 (C-23), 65.0 (C-26), 70.0 (C-5″), 71.9 (C-3), 72.3 (C-3″), 72.4 (C-2″), 73.0 (C-5′), 74.0 (C-4″), 75.7 (C-4′), 76.4 (C-2′), 80.4 (C-6), 82.6 (C-16), 84.3 (C-3′), 102.8 (C-1″), 105.1 (C-1′), 112.6 (C-22). ESI-HRMS m/z 741.4438 $[M + H]^+$ (calculated for $C_{39}H_{65}O_{13}$, 741.4425, Δppm = 1.8).

Compound ***5****, (22R,23S,25S)-3β,6α,23-Trihydroxy-5α-spirostane-6-O-β-D-xylopyranosyl-(1→3)-O-β-D-quinovopyranoside.* ^{1}H NMR (CD_3OD, 500 MHz) δ 0.69 (1H, td, J = 11.5, 4.2 Hz, H-9), 0.77 (3H, d, J = 6.6 Hz, Me-27), 0.81 (3H, s, Me-18), 0.87 (3H, s, Me-19), 0.97 (1H, m, H-7b), 1.03 (1H, m, H-1b), 1.09 (3H, d, J = 7.0 Hz, Me-21), 1.14 (2H, m, H-4b, H-12b), 1.16 (1H, m, H-5), 1.18 (1H, m, H-14), 1.27 (3H, d, J = 6.0 Hz, Me-6′), 1.27 (1H, m, H-15b), 1.32 (1H, m, H-11b), 1.41 (1H, m, H-2b), 1.55 (1H, m, H-11a), 1.61 (1H, m, H-24b), 1.65 (1H, m, H-8), 1.67 (1H, m, H-24a), 1.69 (1H, m, H-17), 1.71 (1H, m, H-1a), 1.75 (2H, m, H-2a, H-12a), 1.98 (1H, m, H-15a), 2.05 (1H, m, H-25), 2.16 (1H, dt, J = 12.4, 4.2 Hz, H-7a), 2.25 (1H, p, J = 7.0 Hz, H-20), 2.40 (1H, dd, J = 10.4, 4.2 Hz, H-4a), 3.05 (1H, t, J = 9.1 Hz, H-4′), 3.23 (1H, t, J = 11.1 Hz, H-5″b), 3.26 (1H, dd, J = 9.2, 7.5 Hz, H-2″), 3.32 (1H, m, H-5′), 3.33 (1H, m, H-3″), 3.35 (1H, m, H-2′), 3.36 (1H, t, J = 11.6 Hz, H-26b), 3.40 (1H, m, H-6), 3.43 (1H, t, J = 9.1 Hz, H-3′), 3.46 (2H, m, H-3, H-26a), 3.49 (1H, m, H-4″), 3.52 (1H, t, J = 2.8 Hz, H-23), 3.90 (1H, dd, J = 11.1, 5.4 Hz, H-5″a), 4.32 (1H, d, J = 7.8 Hz, H-1′), 4.45 (1H, td, J = 7.7, 5.5 Hz, H-16), 4.48 (1H, d, J = 7.5 Hz, H-1″). ^{13}C NMR (CD_3OD, 126 MHz) δ 13.8 (C-19), 16.7 (C-18), 17.0 (C-21), 17.4 (C-27), 18.2 (C-6′), 22.0 (C-11), 25.0 (C-25), 31.9 (C-2), 32.7 (C-4), 33.0 (C-15), 35.3 (C-8), 37.6 (C-10, C-24), 38.5 (C-1), 40.7 (C-12), 41.6 (C-7), 41.7 (C-20), 42.1 (C-13), 51.8 (C-5), 55.1 (C-9), 57.5 (C-14), 65.7 (C-17), 67.1 (C-5″), 67.5 (C-26), 71.0 (C-4″), 71.1 (C-23), 71.8 (C-3), 72.7 (C-5′), 75.2 (C-2′), 75.3 (C-4′, C-2″), 77.7 (C-3″), 80.3 (C-6), 82.4 (C-16), 87.7 (C-3′), 104.8 (C-1′), 106.0 (C-1″), 110.0 (C-22). ESI-HRMS m/z 727.4292 $[M + H]^+$ (calculated for $C_{38}H_{63}O_{13}$, 727.4269, Δppm = 3.2).

Compound ***6****, Paniculonin A.* ^{1}H NMR (CD_3OD, 500 MHz) δ 0.70 (1H, td, J = 11.4, 3.7 Hz, H-9), 0.86 (3H, s, Me-18), 0.88 (3H, s, Me-19), 0.96 (1H, m, H-7b), 0.96 (3H, d, J = 7.0 Hz, Me-21), 1.03 (1H, t, J = 14.3 Hz, H-1b), 1.11 (3H, d, J = 6.7 Hz, Me-27), 1.14 (1H, m, H-4b), 1.17 (2H, m, H-5, H-12b), 1.19 (1H, m, H-14), 1.27 (3H, d, J = 6.1 Hz, Me-6′), 1.33 (1H, m, H-11b), 1.40 (1H, m, H-15b), 1.41 (1H, m, H-2b), 1.55 (1H, brd, J = 13.7 Hz, H-11a), 1.68 (2H, m, H-8, H-24b), 1.72 (1H, m, H-1a), 1.75 (1H, m, H-12a), 1.76 (2H, m, H-2a, H-17), 1.89 (1H, m, H-25), 1.90 (1H, m, H-24a), 1.95 (1H, m, H-15a), 2.17 (1H, dt, J = 12.6, 4.4 Hz, H-7a), 2.40 (1H, brd, J = 11.3 Hz, H-4a), 2.53 (1H, p, J = 7.0 Hz, H-20), 3.05 (1H, t, J = 9.0 Hz, H-4′), 3.19 (1H, d, J = 11.4 Hz, H-26b), 3.23 (1H, t, J = 11.2 Hz, H-5″b), 3.26 (1H, dd, J = 9.1, 7.6 Hz, H-2″), 3.31 (1H, m, H-5′), 3.32 (1H, m, H-3″), 3.34 (1H, m, H-2′), 3.40 (1H, m, H-6), 3.43 (1H, t, J = 9.0 Hz, H-3′), 3.46 (1H, m, H-3), 3.50 (1H, m, H-4″), 3.66 (1H, dd, J = 10.3, 5.2 Hz, H-23), 3.85 (1H, d, J = 11.0 Hz, H-26a), 3.90 (1H, dd, J = 11.2, 5.4 Hz, H-5″a), 4.32 (1H, d, J = 7.8 Hz, H-1′), 4.44 (1H, q, J = 7.4 Hz, H-16), 4.48 (1H, d, J = 7.6 Hz, H-1″). ^{13}C NMR (CD_3OD, 126 MHz) δ 13.3 (C-19), 13.8 (C-21), 16.5 (C-18), 17.1 (C-27), 17.7 (C-6′), 21.6 (C-11), 30.9 (C-25), 31.4 (C-2), 32.0 (C-15), 32.1 (C-4), 34.5 (C-8), 35.5 (C-24), 36.6 (C-20), 37.2 (C-10), 37.9 (C-1), 40.8 (C-12), 41.2 (C-7), 42.0 (C-13), 51.2 (C-5), 54.6 (C-9), 56.9 (C-14), 62.6 (C-17), 63.5 (C-23), 64.7 (C-26), 66.5 (C-5″), 70.6 (C-4″), 71.3 (C-3), 72.3 (C-5′), 74.6 (C-2′), 74.8 (C-2″), 74.9 (C-4′), 77.1 (C-3″), 79.7 (C-6), 82.1 (C-16), 87.2 (C-3′), 104.3 (C-1′), 105.5 (C-1″), 112.0 (C-22). ESI-HRMS m/z 727.4302 $[M + H]^+$ (calculated for $C_{38}H_{63}O_{13}$, 727.4269, Δppm = 4.5).

Aglycone 7a/7b. *Solanolide* ***7a****.* ^{1}H NMR (CD_3OD, 500 MHz) δ 0.73 (1H, m, H-9), 0.73 (3H, s, Me-18), 0.85 (3H, s, Me-19), 0.95 (1H, m, H-7b), 1.03 (2H, m, H-1b, H-5), 1.17 (2H, m, H-4b, H-12b), 1.21 (1H, m, H-14), 1.28 (3H, d, J = 8.0 Hz, Me-21), 1.36 (1H, m, H-11b), 1.41 (1H, m, H-2b), 1.47 (1H, m, H-15b), 1.58 (1H, m, H-11a), 1.63 (1H, m, H-8), 1.72 (1H, m, H-1a), 1.76 (1H, m, H-2a), 1.81 (1H, m, H-12a), 1.97 (1H, m, H-7a), 1.98 (1H, m, H-17), 2.18 (1H, m, H-4a), 2.30 (1H, m, H-15a), 2.61 (1H, m, H-20), 3.34 (1H, m, H-6), 3.49 (1H, m, H-3), 5.03 (1H, td, J = 7.8, 4.6 Hz, H-16). ^{13}C NMR (CD_3OD, 126 MHz) δ 13.6 (C-19), 13.9 (C-18), 17.9 (C-21), 21.4 (C-11), 31.6 (C-2), 32.8 (C-4), 33.8 (C-15), 34.7 (C-8), 37.4 (C-20), 37.6 (C-10), 38.3 (C-1), 38.7 (C-12), 42.2 (C-13), 42.3 (C-7), 52.6 (C-5), 54.9 (C-9), 55.4 (C-14), 59.9 (C-17), 69.6 (C-6), 71.7 (C-3), 84.6 (C-16), 183.9 (C-22). ESI-HRMS m/z 363.2530 $[M + H]^+$ (calculated for $C_{22}H_{3543}$, 363.2535, Δppm = 1.4).

Solanolide-20-epimer ***7b****.* ^{1}H NMR (CD_3OD, 500 MHz) δ 0.73 (1H, m, H-9), 0.81 (3H, s, Me-18), 0.85 (3H, s, Me-19), 0.95 (1H, m, H-7b), 1.03 (2H, m, H-1b, H-5), 1.17 (2H, m, H-4b, H-12b), 1.21 (1H, m, H-14), 1.30 (3H, d, J = 8.1 Hz, Me-21), 1.36 (1H, m, H-11b), 1.41 (1H, m, H-2b), 1.47 (1H, m, H-15b), 1.58 (1H, m, H-11a), 1.63 (1H, m, H-8), 1.72 (1H, m, H-1a), 1.76 (1H, m, H-2a), 1.81 (1H, m, H-12a), 1.97 (1H, m, H-7a), 2.18 (1H, m, H-4a), 2.30 (1H, m, H-15a), 2.31 (1H, m, H-17), 2.97 (1H, m, H-20), 3.34 (1H, m, H-6), 3.49 (1H, m, H-3), 4.83 (1H, td, J = 7.3, 3.7 Hz, H-16). ^{13}C NMR (CD_3OD, 126 MHz) δ 10.3 (C-21), 13.5 (C-19), 14.0 (C-18), 21.4 (C-11), 31.6 (C-2), 32.8 (C-4), 33.8 (C-15), 34.7 (C-8), 37.6 (C-10), 38.3 (C-1), 38.7 (C-20), 38.7 (C-12), 42.2 (C-13), 42.3 (C-7), 52.6 (C-5), 54.9 (C-9), 55.4 (C-14), 56.6 (C-17), 69.6 (C-6), 71.7 (C-3), 84.1 (C-16), 182.2 (C-22). ESI-HRMS m/z 363.2522 $[M + H]^+$ (calculated for $C_{22}H_{3543}$, 363.2535, Δppm = 3.6).

Animals. *Zebrafish.* Adult zebrafish (AB strain) were reared at 28 °C on a 14/10 h light/dark cycle according to standard aquaculture conditions. Eggs were collected following natural spawning, sorted, and raised in 0.3× Danieau's solution (1.5 mM HEPES, pH 7.6, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, and 0.18 mM $Ca(NO_3)_2$) under constant light conditions in an incubator set at 28 °C until 6 or 7 dpf. All zebrafish experiments carried out were approved by the Ethics Committee of the University of Leuven.

Evaluation of Anticonvulsant Activity. *Automated Larval Zebrafish-PTZ Assay.* A 96-well plate was prepared with one 7 dpf zebrafish larva placed per well, and excess larval medium replaced with 100 μL of either control or sample solution. The prepared plate was placed in a dark box inside an incubator, set at 28 °C, for 18 h, and subsequently inspected per well for signs of toxicity (e.g., irregular heart-rate, loss of posture, edema, necrosis, delayed startle or touch response). The maximum tolerated concentration (MTC) was designated as the highest sample concentration that did not elicit any signs of toxicity in 6/7 dpf larvae after 18 h of exposure.[11] Upon addition of 100 μL of 20 mM PTZ per well, the plate was positioned in the zebrafish tracking box with infrared video-recording equipment interfaced to a desktop PC (Zebrabox Viewpoint, Lyon, France), and the larvae allowed to habituate for 5 min before recording for 30 min, with movement values determined at 5 min intervals. Tracking data was exported into Excel format and processed as such before statistical analysis via GraphPad Prism v.5 for Windows. Each tracking data set was normalized against the PTZ-only control values (set at 100%) within each set, with each subsequent replicate set pooled before two-way ANOVA with Bonferroni post hoc analysis.

***Xenopus laevis* GABA$_A$ Receptor Overexpression System.** *Oocyte Preparation and Injection.* The use of animals and all experimental procedures were approved by local authorities (Regierungspraesidium Tuebingen, Tuebingen, Germany). Oocytes were obtained from the Institute of Physiology I, Tübingen. Preparation of oocytes for recordings included treatment with collagenase (1 mg/mL of type CLS II collagenase, Biochrom KG, Berlin, Germany) in OR2 solution (mM: 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, and 5 HEPES, pH 7.6), followed by thorough washing and storing at 16 °C in Barth solution (mM: 88 NaCl, 2.4 $NaHCO_3$, 1 KCl, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, and 5 Tris/HCl, pH 7.4 with NaOH) supplemented with 50 μg/mL gentamicin (Biochrom KG, Germany). For injection and recording, oocytes were plated in 96-well plates. All cRNA concentrations were adjusted to 800 ng/subunit, and

70 nL of the $\alpha1\beta2\gamma2s$ cRNA mixed in a 1:1:2 ratio was injected in each oocyte using robooinject (Multi Channel Systems, Reutlingen, Germany). Recordings were done at day 3 after injection. Amplitudes of interest for all currents recorded from the same oocyte were normalized to the first GABA pulse.

Automated Oocyte Two-Microelectrode Voltage Clamp. The currents in oocytes were recorded at room temperature (20−22 °C) using roboocyte2 (Multi Channel Systems, Reutlingen, Germany). Prepulled and prepositioned intracellular glass microelectrodes had a resistance of 0.3−1 MΩ when filled with 1 M KCl/1.5 M KAc. The bath solution was ND96 (mM: 93.5 NaCl, 2 KCl, 1.8 CaCl2, 2 MgCl2, and 5 Hepes; pH 7.5). All compounds were applied for 15 s followed by 5 min washout with ND96.

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Conceived and designed the study: A.D.C., J.-L.W.; performed zebrafish experiments: O.E.M.B.; performed phytochemistry experiments: S.C., L.M., W.K.; performed electrophysiology experiments: S.M., M.B.; analyzed data: E.F.Q., H.L., C.V.E., P.A.M.d.W.; wrote the manuscript: O.E.M.B., S.C., A.D.C., J.-L.W; provided research materials: F.M.D., A.L.H.

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Notes

The authors declare no competing financial interest.

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