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Design of potent inhibitors for *Schistosoma japonica* glutathione *S*-transferase

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Abstract—We implemented both structure-based drug design and the concept of polyvalency to discover a series of potent and unsymmetrical *Schistosoma japonicum* glutathione *S*-transferase (*Sj*GST) inhibitors **10**–**12**. This strategy achieved not only an excellent enhancement (10- to 490-fold) in the inhibitory potency, compared to the monofunctional analogues **1**–**5**, but was also an effective modification by selecting a hydrophobic moiety with a flexible linker. The designed compounds with a low micromolar hit demonstrate special values in refining the new generation of *Sj*GST inhibitors. The stoichiometry of the binding is one inhibitor molecule per *Sj*GST monomer via isothermal titration calorimetric measurement.

1. Introduction

The glutathione *S*-transferase from the helminthes *Schistosoma japonicum* (*Sj*GST) catalyzes glutathione (GSH) conjugations by facilitating the nucleophilic attack of the sulfhydryl group on various xenobiotics.[1–7] By forming less toxic and more soluble GSH conjugates,[8–13] the harmful electrophiles are readily exported and excreted from the cell. Since this mechanism is the parasite's primary defense system, reduction of the *Sj*GST activity could potentially diminish the worm's ability to withstand electrophilic and oxidative damage resulting from environmental stress and drug administration. In fact, it has been recently reported on the basis of crystallographic data that the major antischistosomal drug, praziquantel,[14] directly binds to *Sj*GST and might have the potential to block the entrance of substrates,[15] even though in vitro study demonstrated that praziquantel does not inhibit *Sj*GST activity[16] using the conventional CDNB (1-chloro-2,4-dinitrobenzene) assay. Nonetheless, the administration of artemether[17] or oltipraz[18,19] resulted in a time- and dose-dependent decrease of the activity of schistosome GST. Thus, this enzyme draws great attraction as a drug target against schistosomiasis,[20–27] a tropical disease infecting 200 million people and causing 280 thousand deaths annually. Although numerous inhibitors for cytosolic and other parasitic GSTs are known,[28–36] to our knowledge, very few are reported for *Sj*GST.[37,38] In the present study, we describe the discovery and synthesis of potent inhibitors of *Sj*GST capable of occupying the active site of the enzyme by a structure-based drug design approach. Furthermore, the feasibility of the inhibitor binding to the dimer interface of *Sj*GST is examined.

2. Results and discussion

A series of GSH analogues (**1**–**5**) were first synthesized with the key Cys modified into different groups, that is, a Ser, a Thr, a β-cyanoalanine, a (*S*)-2-amino-4-cyanobutyric acid, and a L-glutamyl methyl ester (Scheme 1). The IC_{50} values were determined using CDNB assay[39] and the inhibition data are summarized in Table 1. Among the compounds tested, cyano glutathione **3** showed the smallest IC_{50} value of 147 μM. Methyl or methylene groups appearing at γ position make poor inhibitors such as compounds **2**, **4**, and **5**. Suggested by molecular modeling (see Section 4), compound **2**, with an extra methyl substitution, gave much poor inhibition compared to **1**, presumably due to van der Waals strain resulting from the methyl group and the indole side chain of Trp8 in the active site (Fig. 1A). Therefore, compound **1**, γ-L-glutamyl-L-seryl-glycine (GOH), was chosen as our glutathionyl building block.

Keywords: Drug design; Molecular modeling; Epiandrosterone; *Schistosoma japonica* glutathione *S*-transferase; Inhibitors.
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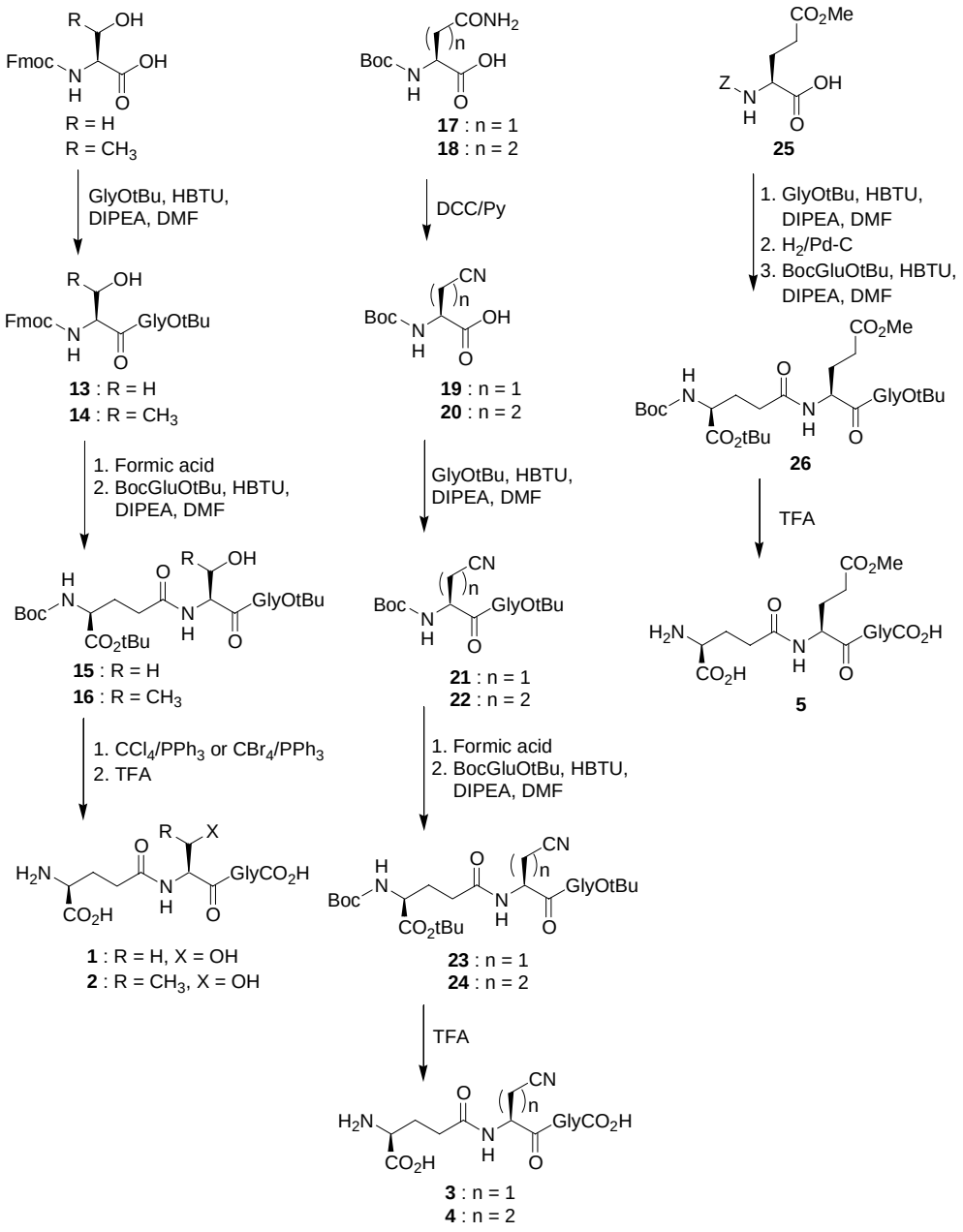
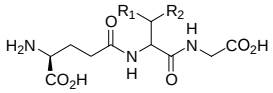


Scheme 1. Synthesis of the monofunctional analogues **1**–**5**.

Table 1. Monovalent inhibitors for *Sj*GST



Compound	R_1	R_2	IC_{50} (μM)
1	H	OH	395
2	CH_3	OH	>1500
3	CN	H	147
4	CH_2CN	H	>3000
5	CH_2CO_2Me	H	774

To understand the existing binding modes of *Sj*GST, crystal structures of enzyme with *S*-hexyl glutathione (1M9A), *S*-2-iodobenzyl glutathione (1M9B)[40] or praziquantel (1GTB)[15,16] were used to identify the binding cavity of the enzyme. It was realized that *S*-hexyl glutathione (HEX) and *S*-2-iodobenzyl glutathione (IBZ) occupied both glutathione binding site (G site) and electrophile binding site (H site), while praziquantel (PZQ) was binding at the dimer interface. Since their positions within the enzyme were not far from each other (Fig. 1B), our plan was to construct multivalent inhibitors, occupying multiple sites of *Sj*GST, to result in a more favorable binding than the monofunctional analogues. The optimizations can be achieved by exploring the binding domains and the tether between them. Experimentally, Remoué et al. confirmed a specific binding of testosterone to *Schistosoma haematobium* glutathione *S*-transferase (*ShGST*) with a high K_d value of affinity at 2.57×10^{-7} M.[41] Other steroids such as

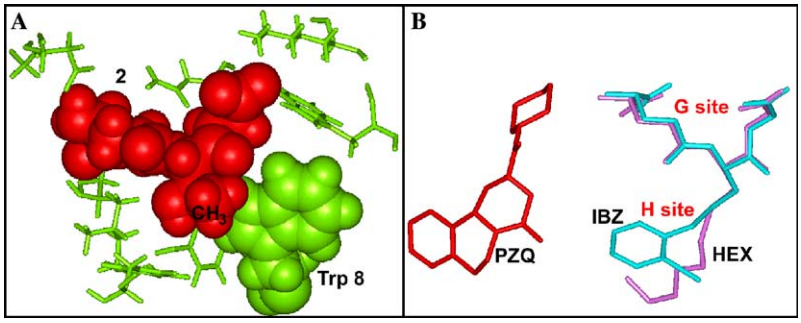
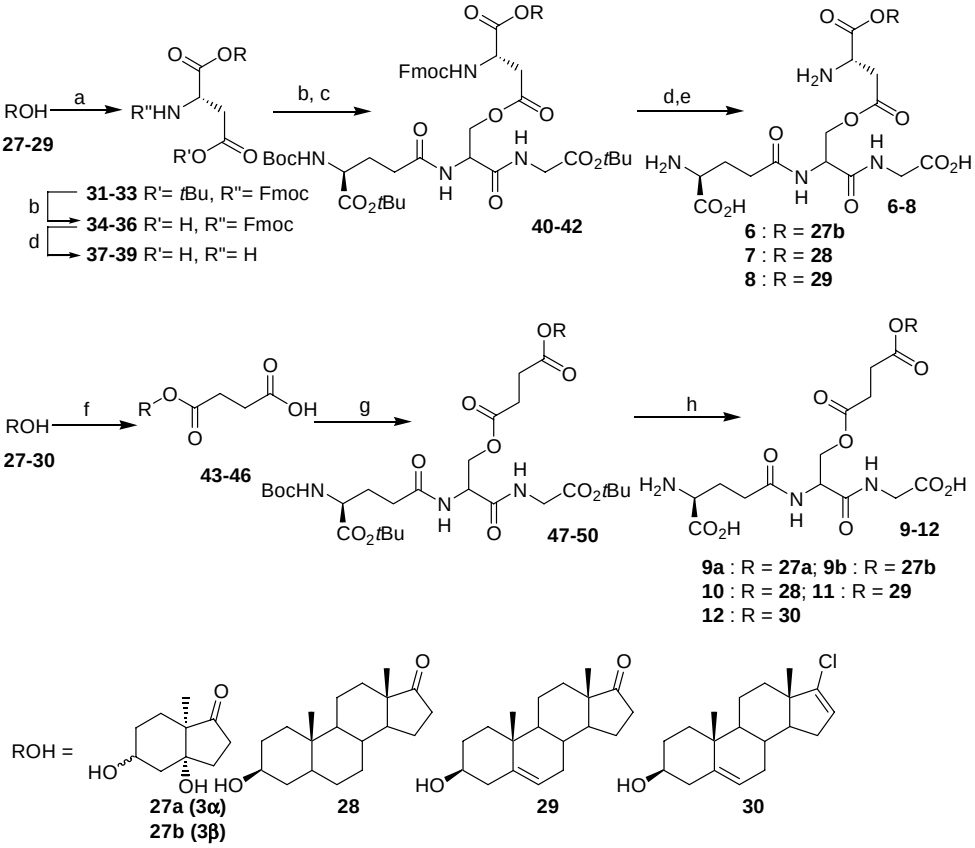


Figure 1. (A) Steric hindrance resulting from methyl group of Thr in compound **2** (red) and the residue Trp 8 of *Sj*GST (green); (B) The relative positions of the inhibitors, that is, *S*-hexyl glutathione (HEX, purple), *S*-2-iodobenzyl glutathione (IBZ, cyan), and praziquantel (PZQ, red) occupying the G, H sites and the dimer interface of *Sj*GST.

Δ5-androstene-3,17-dione,[42] 3β-(iodoacetoxy)dehydroisoandrosterone,[43] 17β-estradiol-3,17-disulfate,[43] and 17β-iodoacetoxy-estradiol-3-sulfate[44] were reported to have a binding interaction with rat GST 1-1 or human GST A3-3. Due to the lack of X-ray structure, however, the precise binding site of steroid inside specific GST is still unclear. Because steroids are known to bind with specific GST,[41–46] existing steroidal analogues are chosen as the hydrophobic moieties to enhance the binding affinity between inhibitor and *Sj*GST. Prior to testing the multivalent inhibitor potency, we prepared two epiandrosterone derivatives, **38** and **44**, with different linkers (aspartic acid and succinic acid). Compound **38** was synthesized by the esterification of **28** and Fmoc-Asp (O-*t*-Bu)-OH followed by subsequent deprotection of *t*-Bu and Fmoc groups (Scheme 2). Derivative **44** was prepared by treating **28** with succinic anhydride in the presence of pyridine and 4-dimethylaminopyridine (DMAP). Compound **38** was found to have an IC_{50} of 128 μM, while **44** had an IC_{50} of 294 μM.

Having the building blocks in hand, compounds **6–8** were prepared with an Asp linkage to connect GOH and **27–29**, respectively. The synthetic pathway is outlined in Scheme 2. L-Aspartic acid was first esterified to **27–29** separately then to GOH. Compounds **6–8** were obtained after removal of the Fmoc and then acidolytic cleavage of the remaining *t*-Bu and Boc groups. All products were purified by lipophilic Sephadex LH-20 and reverse-phase HPLC. The indene substructure of **6**



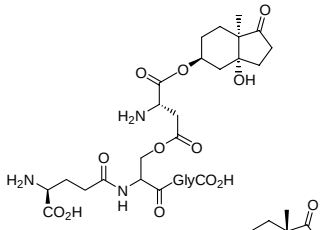
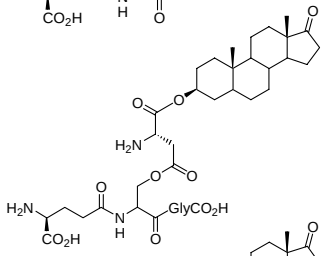
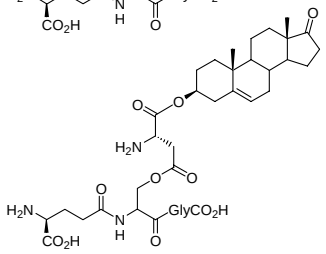
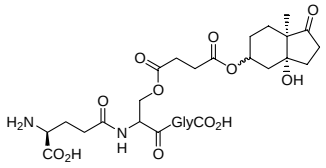
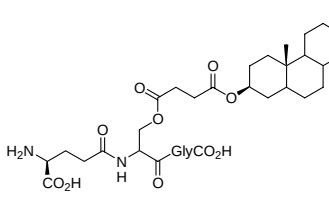
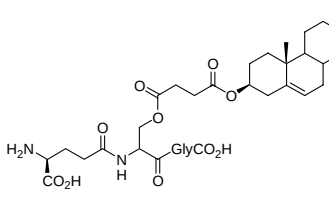
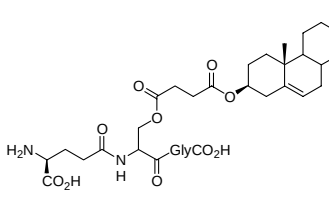
Scheme 2. Synthesis of the bifunctional analogues **6–12**. Reagents and conditions: (a) Fmoc-Asp(O-*t*-Bu)-OH, DMAP, DCC, CH_2Cl_2, 25 °C; (b) TFA, CH_2Cl_2, 0 °C; (c) **15**, DMAP, DCC, CH_2Cl_2, 25 °C; (d) DBU, CH_2Cl_2, rt; (e) TFA, 2% H_2O, 0 °C; (f) succinic anhydride, DMAP, pyridine, rt; (g) **15**, DMAP, DCC, CH_2Cl_2, 25 °C; (h) TFA, 2% H_2O, 0 °C.

was chosen because it was analogous to the corresponding epiandrosterone rings C and D. However, the indene substructure of **6** with an extra hydroxyl group and the absence of classical steroids rings A and B in comparison with the epiandrosterone moiety of **7** or **8** should reduce the potential of hydrophobicity and might have a somewhat lower affinity for the electrophile binding site (H site). As expected, compounds **7** and **8** display a 10- to 16-fold improvement in potency over **1**, while **6** exhibits a 2-fold reduction (Table 2), indicating that the tight-binding inhibitors need an epiandrosterone moiety. In contrast, current observation suggests that attachment of the indene group to **1** through an Asp linker does not promote proper binding in the active site of *Sj*GST and lead to the poor activity.

In addition, compounds **9**–**11** were prepared using succinyl linkage between GOH and **27**–**29** to test the influence of the linker (Scheme 2). The significance is demonstrated by comparing compounds **6**–**8** and **9**–**11**—the linkers are of the same length, but compounds having a succinyl tether yield an IC_{50} value about 3-fold lower (Table 2). All of the tight-binding inhibitors contained both steroid and succinyl linker. For example, **10** and **12** display IC_{50} values of 8 and 7 μM, representing a 112- to 128-fold enhancement over **6**. Furthermore, compounds **10**–**12** are at least 10- to 490-fold more potent than monofunctional compounds **1**–**5** and 9- to 42-fold more effective than compounds **38** and **44**.

In order to visualize the feasible binding mode between inhibitors and enzyme, we carried out a molecular modeling study of docking compound **10** to X-ray structure of *Sj*GST. First, potential binding pockets were identified using the ActiveSite_Search module within the InsightII program (see Section 4). Two cavities were found near the G site and were used to dock the epiandrosterone moiety. After energy minimization of enzyme–ligand complexes, two different and stable conformations were obtained. One of the complexes had the inhibitor occupying the same sites as HEX, IBZ, and PZQ (Fig. 2, conformation 1). The epiandrosterone moiety was found at the dimer interface with hydrophobic interaction to both Tyr 104 residues from each monomer (Fig. 3). The second conformation illustrated that the epiandrosterone moiety located near the H site and the cavity near surface (Fig. 2, conformation 2). If the inhibitor adopted the second conformation, one GST dimer would be able to bind two inhibitors. On the other hand, if the inhibitor occupied the dimer interface just like PZQ, one GST dimer could only interact with one inhibitor. In addition, PZQ would interfere with this interaction. In order to verify which model is more appropriate, isothermal titration calorimetry (ITC) and kinetic study were performed. Figure 4 demonstrates the thermodynamics of **10** binding to *Sj*GST monomer in a 0.96:1 stoichiometry. The ITC result unambiguously suggests that **10** did not bind to the dimer interface because occupation of the single dimer interface would simultaneously preclude binding by another inhibitor. As mentioned above, PZQ is known to bind to the dimer interface without interfering with *Sj*GST's catalytic ability. More significant support was disclosed from kinetic effect of PZQ on inhibition of *Sj*GST by compound **11** (Fig. 5). The IC_{50} value of **11** (14 μM) for *Sj*GST was not affected by up to 250 μM PZQ. This strongly indicates that PZQ does not compete with **11** for *Sj*GST binding at the dimer interface. Both direct evidences favor the second conformation (Fig. 2, conformation 2) and suggest the ligands

Table 2. Bivalent inhibitors for *Sj*GST

Compound	#	IC_{50} (μM)
	**6**	896
	**7**	24
	**8**	40
	**9a** (α-isomer) **9b** (β-isomer)	277 230
	**10**	8
	**11**	14
	**12**	7

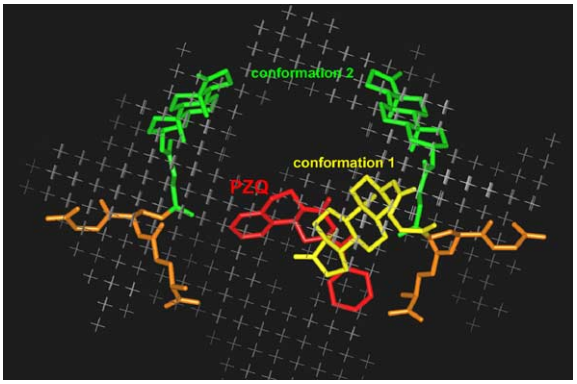


Figure 2. Visualization of the binding cavities of *Sj*GST with the bound inhibitor **10** in two different binding modes (conformation 1 and conformation 2). The bound inhibitor **10** in conformation 1, occupying the same sites as HEX, IBZ, and PZQ (red), is shown in yellow. The conformation 2, occupying the G site and the cavity near surface, is depicted in green. The symmetric structure of conformation 2 (left portion of this picture) is displayed in the other *Sj*GST monomer.

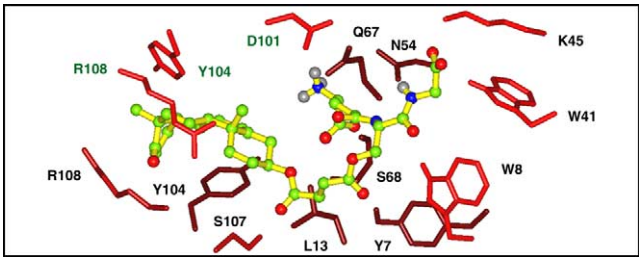


Figure 3. Interactions between **10** (conformation 1) and *Sj*GST. The residues from the other monomer were labeled green.

(**10**–**12**) might interact with the G and H sites of *Sj*GST during inhibitory binding. Interestingly, both conformations have succinyl linker located in a hydrophobic environment surrounded by Ile 10, Tyr 111 or Leu 13. This makes additional NH_3^+ group from the Asp linker less favorable. A more detailed picture of the interaction between ligand and *Sj*GST requires an X-ray structure determination of the complex and the putative steroid binding site will then be revealed.

3. Conclusions

In summary, we have successfully designed novel and unsymmetrical inhibitors of *Sj*GST. These compounds demonstrate the first example to enhance a positive interaction toward *Sj*GST by manipulating the hydrophobicity of the linker and using the steroid moiety as the inhibitor building block. The present results suggest that further development of a new generation of *Sj*GST inhibitors is quite likely to be discovered.

4. Experimental

4.1. Materials and general methods

Chemicals were obtained from Aldrich. All amino acids were purchased from Advanced Chemtech. Restriction

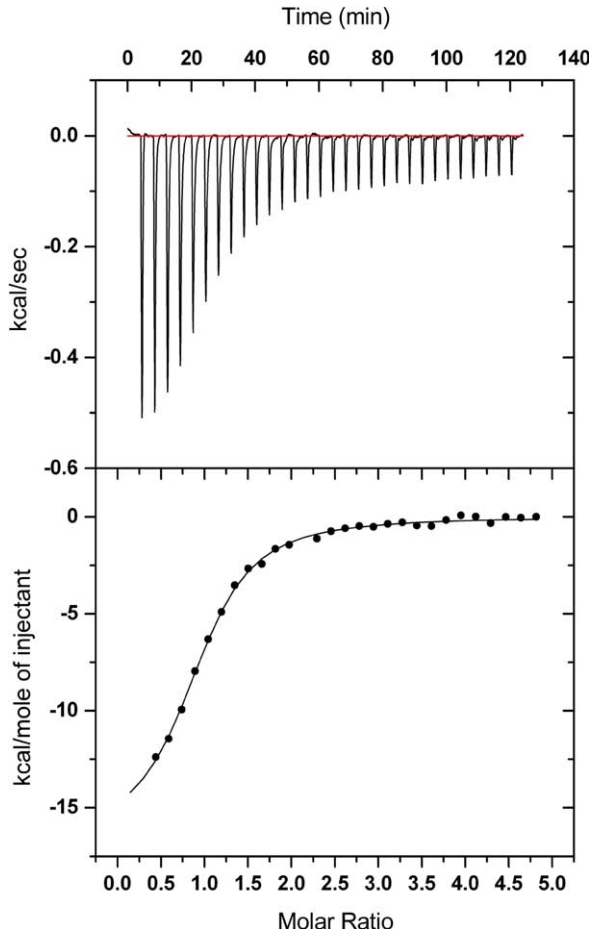


Figure 4. Isothermal calorimetric titration of *Sj*GST with inhibitor **10**. Top, the exothermic binding effect was observed from the addition of 10 μL aliquots of 125 μM **10** to a 6 μM solution of *Sj*GST. The concentration was expressed as *Sj*GST monomer; bottom, binding isotherm corresponding to the integrated heats in the top panel represents the best fitted curve using ORIGIN 5.0 software.

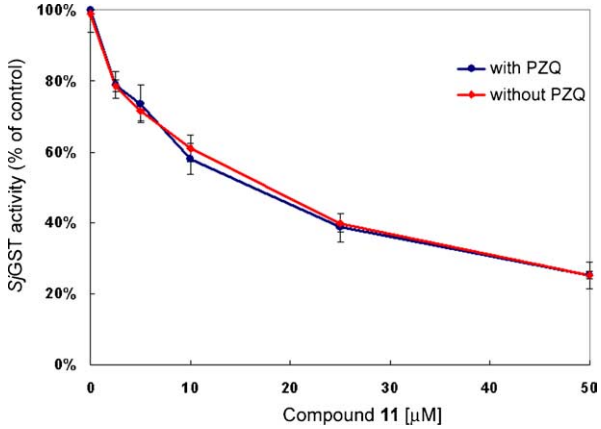


Figure 5. Inhibition of *Sj*GST activity by inhibitor **11** in the presence or absence of PZQ at a concentration of 100 μM. Each data point represents the mean of at least three experiments plus or minus standard error. Activity is expressed as percentage of uninhibited control.

enzymes were from Promega or New England Biolabs. Minipreps and gel extraction DNA purification kits were from Qiagen. Plasmids pET-15b and pGEX-4T-1

were from Novagen and Amersham Pharmacia Biotech, respectively. Oligonucleotide synthesis and DNA sequencing reactions were conducted by the MDBio. Hi-Trap affinity column was purchased from Amersham Pharmacia Biotech.

^{1}H and ^{13}C NMR spectra were recorded with Bruker AMX400 or 500 MHz instruments. Proton chemical shifts (δ) are reported in parts per million (ppm) relative to the methine singlet at 7.24 ppm for the residual $CHCl_3$ in the deuteriochloroform or the methyl pentet at 3.30 ppm for the residual CHD_2OD in the methanol-d_4. Carbon chemical shifts are reported in parts per million relative to the internal ^{13}C signals in $CDCl_3$ (77.0 ppm) and CD_3OD-d_4 (49.0 ppm). Mass spectra were obtained with a FAB JMS-700 double focusing mass spectrometer (JEOL, Tokyo, Japan), MALDI Voyager DE-PRO (Applied Biosystem Houston, USA), and ESI Finnigan LCQ mass spectrometer (Thermo Finnigan, San Jose, CA, USA) in negative mode. The purity of compounds was determined by reverse-phase analytical HPLC (Waters 2695 System with a 996 PDA detector), using Vydac 201SP54 C18 column (250×4.6 mm, 5 μm) and Sephadex LH-20 (170×10 mm) column. The separation procedure was performed using H_2O/0.1% TFA (A), 89.95% CH_3CN/10% H_2O/0.05% TFA (B), and 89.9% H_2O, 10% CH_3CN/0.1% TFA (C) as eluents. Twelve different methods were used:

Method 1: Vydac 201SP54 C18 column, flow rate 2 mL/min, using the following gradient: from 100% A, 15 min isocratic.
Method 2: Sephadex LH-20 column, flow rate 0.75 mL/min, using the following isocratic: 100% H_2O.
Method 3: Vydac 201SP54 C18 column, flow rate 2 mL/min, using the following gradient: from 100% A, 12 min isocratic, linear to 90% B in 8 min, then linear to 100% A in 7 min, then isocratic.
Method 4: Sephadex LH-20 column, flow rate 0.75 mL/min, using the following isocratic: 50% H_2O, 50% MeOH.
Method 5: Vydac 201SP54 C18 column, flow rate 2 mL/min, using the following gradient: from 98% A, 5 min isocratic, linear to 70% A in 15 min, then linear to 50% A in 5 min, then linear to 100% A, then isocratic.
Method 6: Vydac 201SP54 C18 column, flow rate 1 mL/min, using the following gradient: from 100% C, 5 min isocratic, linear to 95% C in 10 min, then linear to 100% B, then isocratic.
Method 7: Sephadex LH-20 column, flow rate 0.75 mL/min, using the following isocratic: 100% MeOH.
Method 8: Vydac 201SP54 C18 column, flow rate 2 mL/min, using the following gradient: from 60% C, linear to 40% C in 20 min, then linear to 90% B in 5 min, then linear to 60% C in 5 min, then isocratic.
Method 9: Vydac 201SP54 C18 column, flow rate 1 mL/min, using the following gradient: from 70% C, linear to 55% C in 10 min, then linear to 100% B in 4 min, 1 min isocratic, then linear to 70% C in 14 min, then isocratic.
Method 10: Vydac 201SP54 C18 column, flow rate 1 mL/min, using the following gradient: from 90% C, linear to 85% C in 20 min, then linear to 20% C in 5 min, 1 min isocratic, then linear to 90% C in 6 min, then isocratic.
Method 11: Vydac 201SP54 C18 column, flow rate 1 mL/min, using the following gradient: from 50% C, linear to 40% C in 5 min, then linear to 10% C in 15 min, then linear to 100% B in 5 min, 5 min isocratic, then linear to 50% C in 5 min, then isocratic.
Method 12: Vydac 201SP54 C18 column, flow rate 2 mL/min, using the following gradient: from 30% C, linear to 20% C in 5 min, then linear to 10% C in 20 min, then linear to 30% C in 5 min, then isocratic.

Semipreparative reverse-phase HPLC was conducted on a Vydac 201SP510 C18 column (250×10 mm, 5 μm) using H_2O/0.1% TFA (A) and 89.95% CH_3CN/10% H_2O/0.05% TFA (B) as eluents; detection at 220, 254 nm with a Waters 2487 dual λ absorbance detector.

4.2. Subcloning, expression, and purification of *Schistosoma japonica* glutathione *S*-transferase (*Sj*GST)

The DNA of glutathione *S*-transferase was amplified by PCR from plasmid pGEX-4T-1 using oligonucleotides (5′–3′): GGAATTCCATATGTCCCCTATACT AG and GCGGGATCCTTATTTTGGAGG having NdeI and BamHI restriction sites. The resulting product was cut by *Nde*I and *Bam*HI, and ligated into pET-15b to yield pET15b-GST. This construct allowed expression of GST in N-terminal fusion with a hexahistidine tag in *Escherichia coli* under control of IPTG inducible T7 promoter. The expression construct was verified by sequence analysis and was transformed into *E. coli* strain BL21. A portion (14 mL) of overnight cell culture was inoculated for each 1 L of culture medium at 37 °C. IPTG was added to a final concentration of 0.4 mM when the OD_{600} reached 1.0 in LB media. The cells were harvested after 4 h by centrifugation at 6000 rpm and stored at −80 °C. *E. coli* cells (from 1 L) were resuspended in 30 mL of 20 mM Tris–HCl, pH 7.4, 0.2 mM DTT at 4 °C. The suspension was passed twice through a French press. The pressure should not exceed 11,000 psi. The resulting cell lysate was centrifuged at 12,000 rpm for 20 min. To the supernatant were added 5 M NaCl and 5 M imidazole solution so that the final concentration of NaCl was 500 mM and imidazole was 10 mM. The solution was again centrifuged at 12,000 rpm for 20 min and the supernatant was applied to a fully equilibrated Ni^{2+} chelating column (bed volume 5 mL). The column was washed with 25 mL, 20 mM Tris–HCl, pH 7.4, NaCl 500 mM, and imidazole 10 mM. The bound protein was eluted by applying a gradient of imidazole from 10 mM to 300 mM to the column. Fractions containing GST were pooled and dialyzed against 20 mM Tris–HCl, pH 7.4, and were finally stored in 20% glycerol at −80 °C.

4.3. *Sj*GST assays

Ten nanomoles of *Sj*GST was incubated with 1 mM CDNB in 0.1 M potassium phosphate buffer, pH 6.5, in a total volume of 225 μL at 30 °C for 3 min. The reaction was initiated by adding 25 μL of 10 mM GSH and monitored at 340 nm. To determine the IC_{50}, various concentrations of the inhibitor were incubated with *Sj*GST for 3 min prior to initiation by GSH. The IC_{50} values were

obtained by fitting the data to the equation $V_i = V_0/[(1 + C_{inh})/IC_{50}]$, where C_{inh} is the concentration of the inhibitor.

4.4. Isothermal titration calorimetry

Thermodynamic analysis of the interaction between *Sj*GST and **10** was performed using a VP-ITC microcalorimeter (MicroCal, Inc., Northhampton, MA). The protein (6 μM monomer) solution in the calorimetric cell was titrated with inhibitor **10** (125 μM) solution dissolved in the same buffer (10 mM potassium phosphate, pH 6.5, and 10% DMSO). Both solutions were degassed under vacuum for 10 min. All experiments were performed at 25 °C with a 300 μL injection syringe. The titration solution was injected with a stirring speed of 300 rpm at discrete intervals of 240 s. Titrations were performed by injecting 10 μL of **10** stock into the ITC sample cell containing 6 μM *Sj*GST. The data were integrated and fitted using a one-site binding model with the ORIGIN 5 analysis software (MicroCal).

4.5. Modeling of the inhibitor *Sj*GST complex

Models of the *Sj*GST complexed with the inhibitor molecules, γ-glutamyl-L-threonylglycine (**2**) or epiandrosterone-succinyl-glutathione (**10**), were generated by Discover within InsightII program (Accelrys, San Diego, CA). The starting geometry was taken from the crystallographic coordinates of *Sj*GST and its inhibitors, *S*-hexyl glutathione, from the Protein Data Bank, 1M9A.[40] Compared with the other two *Sj*GST structures, 1M9B and 1GTB, the water molecules, which were 5 Å close to the bound inhibitors, that is, *S*-hexyl glutathione, *S*-2-iodobenzyl glutathione, and praziquantel, were removed. After deleting the *S*-hexyl moiety, hydrogen atoms were added to the glutathione and the protein by insight II automatically. Finally, the sulfur atom in the glutathione was replaced by an oxygen to give γ-glutamyl-L-serylglycine (GOH). The partial charges were assigned using CFF91 force field. This GOH bound GST was then subjected to energy minimization as described below. All heavy atoms were tethered using harmonic restraints and energy minimized using conjugated gradient until the maximum derivative was less than 0.5 kcal/(mol Å). The restraints were then only applied to the backbone of GST and the heavy atoms of GOH. The whole structure was again minimized until the maximum derivative was less than 0.25 kcal/(mol Å). Finally, the restraints were removed and minimized until the maximum derivative was less than 0.1 kcal/(mol Å). This structure, GST–GOH, was used as the starting coordinates for constructing γ-glutamyl-L-threonylglycine bound GST and docking succinyl epiandrosterone moiety in the future steps.

To build γ-glutamyl-L-threonylglycine bound GST, the serine residue in GOH was replaced by a threonine. Residues located beyond 5 Å of the molecule were tethered. The whole complex was then subjected to 1000 steps of energy minimization, 1000 steps of dynamics at 300 K, and finally 1000 steps of energy minimization untill the maximum derivative was less than 0.1 kcal/(mol Å). The resulting structure suggested that the methyl group from Thr has van der Waals strain with the residue Trp 8 (Fig. 1).

Then again, GST–GOH obtained from previous calculation was used as the receptor to dock succinyl epiandrosterone moiety in the binding site. The binding pockets were identified using the ActiveSite_Search function within the Binding_Site module in the insightII program (Accelrys, San Diego, CA). The grid size was set to 1.4 Å. The cutoff size and the site opening were set to default values which were 50 grid points and 7 Å, respectively. GOH moiety was treated as part of the protein. Cavities reported from the calculation were ignored if not close to the G site. This lead to two big pockets for docking succinyl epiandrosterone moiety (Fig. 2). On the other hand, the succinyl epiandrosterone moiety was built on the androsten-3β-ol-17-one taken from the X-ray structure, 1E3R.[47] The succinyl linker was constructed using Builder module within the program. The dihedral angles of the succinyl linker were adjusted such that it resembled the dihedral angles of the *S*-hexyl moiety in the X-ray structure (1M9A). At the first stage simulation, GST and the GOH moieties were fixed. The complex was subjected to 1000 steps of minimization using conjugate gradients. After the minimization, the whole inhibitor was free to move and subjected to another 1000 steps of minimization. This construction generated the first conformation (Fig. 2). To lead epiandrosterone moiety to take the other cavity near the surface, the dihedral angles of the succinyl linker were rotated manually to dock epiandrosterone moiety to the enzyme. Again, after two stages of minimization, conformation 2 was generated (Fig. 2).

4.6. Synthesis and characterization of monofunctional inhibitors

4.6.1. γ-Glutamyl-L-serylglycine (1). Compound **15** (0.14 g, 0.16 mmol) was dissolved in TFA (3 mL) with 2% ddH_2O (60 μL) and the reaction mixture was stirred at 0 °C for 2 h. The TFA solution was then removed on a rotary evaporator and the residue was used membrane (MWCO = 100) to remove salt. The residue was dissolved in water/TFA (100:0.1) and then purified by RP-HPLC (isocratic: 100% A). The product **1** was obtained as a white solid (78 mg, 37%) upon lyophilization. ^{1}H NMR (D_2O, 400 MHz) δ 4.52 (t, J = 5.3 Hz, 1H), 4.04–4.01 (m, 3H), 3.90 (d, J = 5.3 Hz, 2H), 2.62 (td, J = 7.2, 2.3 Hz, 2H), 2.27–2.22 (m, 2H); ^{13}C NMR (D_2O, 100 MHz) δ 174.6, 173.0, 172.3, 61.0, 55.5, 52.7, 41.0, 30.9, 25.5; MS (FAB, *m*/*z*) 292 $(M+H)^+$; HRFABMS calcd for $C_{10}H_{18}O_7N_3$ 292.1145, found 292.1144; Analytical HPLC: Method 1: t_R, 7.99 min; single peak (100% area); Method 2: t_R, 9.10 min (97% area).

4.6.2. γ-Glutamyl-L-threonylglycine (2). The similar pathway used to prepare **1** synthesized this compound. The product was purified by RP-HPLC (isocratic: 100% A) to give **2** (56 mg, 66%) upon lyophilization. ^{1}H NMR (D_2O, 400 MHz) δ 4.44 (d, J = 3.5 Hz, 1H), 4.32 (m, 1H), 4.14–4.04 (m, 3H), 2.73–2.65 (m, 1H), 2.31–2.27 (m, 1H), 1.30 (d, J = 5.1 Hz, 3H); ^{13}C NMR (D_2O,

100 MHz) δ 174.8, 173.1, 172.6, 172.4, 67.0, 59.1, 52.9, 41.1, 31.0, 25.7, 18.7; MS (FAB, *m/z*) 306 $(M+H)^+$; HRFABMS calcd for $C_{11}H_{20}O_7N_3$ 306.1301, found 306.1304; Analytical HPLC: Method 1: t_R, 8.30 min, single peak (100% area); Method 2: t_R, 8.68 min (93% area).

4.6.3. N^5-[(1*S*)-2-[(Carboxymethyl)amino]-1-(cyanomethyl)-2-oxoethyl]glutamine (3). Compound **3** was synthesized from **23** by the similar pathway used to prepare **1**. Tripeptide **3** was purified by RP-HPLC (gradient: 100% A, 12 min isocratic, 8 min to 10% A and 90% B) and was obtained as a white solid (74 mg, 48%) upon lyophilization. ^{1}H NMR (D_2O, 400 MHz) δ 4.88–4.85 (m, 1H), 4.06–4.03 (m, 3H), 3.07 (dd, J = 7.8, 5.5 Hz, 2H), 2.65–2.60 (m, 2H), 2.29–2.23 (m, 2H); ^{13}C NMR (D_2O, 100 MHz) δ 174.5, 173.0, 171.9, 172.0, 118.1, 52.5, 49.4, 41.2, 30.9, 25.4, 20.0; MS (FAB, *m/z*) 301 $(M+H)^+$; HRFABMS calcd for $C_{11}H_{17}N_4O_6$ 301.1148, found 301.1148; Analytical HPLC: Method 3: t_R, 9.26 min (100% area); Method 4: t_R, 8.62 min (98% area).

4.6.4. γ-Glutamyl-5-azanylidyne-L-norvalylglycine (4). Compound **4** was synthesized from **24** by the similar pathway used to prepare **3**. The final product **4** was purified by RP-HPLC (gradient: 100% A, 12 min isocratic, 8 min to 10% A and 90% B) to afford a white solid (43 mg, 50%) upon lyophilization. ^{1}H NMR (D_2O, 400 MHz) δ 4.54 (dd, J = 9.3, 5.1 Hz, 1H), 4.07 (d, J = 3.8 Hz, 2H), 4.01 (t, J = 6.4 Hz, 1H), 2.67 (t, J = 7.1 Hz, 2H), 2.63–2.56 (m, 2H), 2.32 (m, 3H), 2.13–2.07 (m, 1H); ^{13}C NMR (D_2O, 100 MHz) δ 174.6, 173.1, 173.0, 172.4, 120.5, 52.9, 52.6, 41.1, 31.0, 26.6, 25.5, 13.5; MS (FAB, *m/z*) 315 $(M+H)^+$; HRFABMS calcd for $C_{12}H_{19}O_6N_4$ 315.1305, found 315.1299; Analytical HPLC: Method 3: t_R time, 9.26 min, single peak (100% area); Method 2: t_R 8.62 min, single peak (>98% area).

4.6.5. γ-Glutamyl-(γ-methoxycarbonyl)-α-glutamylglycine (5). Compound **5** was synthesized from **26** by the similar pathway used to prepare **3**. The final product **5** was purified by RP-HPLC (gradient: 98% A and 2% B, 5 min isocratic, 15 min to 70% A and 30% B, and then 5 min to 50% A and 50% B) to afford a white solid (30 mg, 50%) upon lyophilization. ^{1}H NMR (D_2O, 400 MHz) δ 4.42 (dd, J = 8.8, 5.7 Hz, 1H), 4.08–3.98 (m, 3H), 3.73 (s, 3H), 2.61–2.53 (m, 4H), 2.26–2.16 (m, 3H), 2.09–2.02 (m, 1H); ^{13}C NMR (D_2O, 100 MHz) δ 157.7, 156.6, 156.1, 155.2, 154.3, 40.6, 40.3, 40.0, 29.3, 19.3, 19.6, 18.6, 15.1, 14.5; MS (FAB, *m/z*) 348 $(M+H)^+$, HRFABMS calcd for $C_{13}H_{22}O_8N_3$ 348.1400, found 348.1400; Analytical HPLC: Method 5: t_R, 12.77 min, single peak (100% area); Method 2: t_R, 8.96 min, single peak (100% area).

4.6.6. *tert*-Butyl-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-serylglycinate (13). Fmoc-Ser-OH (1.63 g, 4.98 mmol), H-Gly-O-*t*-Bu·HCl (876 mg, 5.23 mmol), and HBTU (2.08 g, 5.48 mmol) were dissolved in dry DMF (15 mL) at room temperature under nitrogen. DIPEA (2.61 mL, 14.94 mmol) was added dropwise over a period of 5 min. The resulting solution was stirred at 25 °C for 40 min and the solvent was then removed under reduced pressure. The residue was dissolved in EtOAc, washed with water, dried over Na_2SO_4, and concentrated in vacuo. The product was purified by silica gel chromatography (hexane/EtOAc 7:3 to 6:4) to afford **13** (1.96 g, 90%) as a white solid. TLC (SiO_2, hexane/EtOAc 3:7): R_f = 0.60; mp 124–125 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.80 (d, J = 7.5 Hz, 2H), 7.64 (d, J = 6.2 Hz, 2H), 7.44 (t, J = 7.4 Hz, 2H), 7.34 (td, J = 7.5, 1.0 Hz, 2H), 7.01 (br, 1H), 6.23 (d, J = 7.0 Hz, 1H), 4.46 (d, J = 6.8 Hz, 2H), 4.42 (s, 1H), 4.25 (t, J = 6.9 Hz, 1H), 4.10 (d, J = 9.6 Hz, 1H), 4.00 (d, J = 4.3 Hz, 2H), 3.78 (dd, J = 11.2, 5.6 Hz, 1H), 3.51 (s, 1H), 1.50 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.1, 169.0, 156.5, 143.6, 141.2, 127.6, 127.0, 125.0, 119.9, 82.5, 67.2, 62.9, 55.9, 47.0, 42.1, 27.9; MS (FAB, *m/z*) 441 $(M+H)^+$; HRFABMS calcd for $C_{24}H_{29}N_2O_6$ 441.2026, found 441.2017.

4.6.7. *tert*-Butyl-*N*-(*tert*-butoxycarbonyl)-α-(*tert*-butyl)-γ-glutamyl-L-serylglycinate (15). Compound **13** (1.96 g, 4.45 mmol) was dissolved in CH_2Cl_2 (10 mL). DBU (0.7 mL, 4.67 mmol) was added dropwise and the reaction mixture was stirred at 25 °C for 20 min. The solvent was then removed on a rotary evaporator and the residue was purified by flash chromatography (5:5 hexane/EtOAc to 9:1 CH_2Cl_2/MeOH) to afford the Fmoc-deprotected dipeptide as an oil (0.95 g, 98%). TLC (SiO_2, CH_2Cl_2/MeOH 9:1): R_f = 0.20. The Fmoc-deprotected dipeptide (0.95 g, 4.36 mmol), Boc-Glu-O-*t*Bu (1.19 g, 3.92 mmol), and HBTU (1.65 g, 4.36 mmol) were dissolved in dry DMF (15 mL) at room temperature under nitrogen, and DIPEA (2.28 mL, 13.08 mmol) was added dropwise. The resulting solution was stirred at 25 °C for 40 min and then concentrated in vacuo. EtOAc was added to the reaction residue, which was then washed repeatedly with water. The organic layers were dried over anhydrous Na_2SO_4 and filtered, and the filtrate evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 7:3 to 6:4) to obtain the desired product **15** as a white crystal (1.58 g, 72%). TLC (SiO_2, hexane/EtOAc 3:7): R_f = 0.40; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.29 (br, 1H), 6.96 (d, J = 6.1 Hz, 1H), 5.32 (br, 1H), 4.56–4.52 (m, 1H), 4.09 (br, 1H), 3.96 (dd, J = 11.5, 4.1 Hz, 1H), 3.88 (d, J = 5.5 Hz, 2H), 3.71 (dd, J = 10.6, 3.9 Hz, 1H), 3.51 (s, 1H), 2.32 (t, J = 7.3 Hz, 2H), 2.14 (m, 1H), 1.83 (m, 1H), 1.41–1.38 (m, 18H), 1.25–1.20 (m, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.7, 171.4, 170,9, 168, 9, 155.8, 82,3, 80.0, 62.7, 54.5, 53.3, 42.0, 32.1, 28.7, 28.3, 28.0, 27.9; MS (FAB, *m/z*) 504 $(M+H)^+$; HRFABMS calcd for $C_{23}H_{41}N_3O_9Na$ 526.2741, found 526.2749.

4.6.8. *N*-(*tert*-Butoxycarbonyl)-5-nitrilo-L-norvaline (20). A solution of Boc-L-glutamine (3 g, 12.2 mmol) in 60 mL pyridine and acetic anhydride (1.38 mL, 14.6 mmol) was stirred at room temperature overnight and concentrated. EtOAc was added to the reaction residue, which was then washed repeatedly with 6% HCl and brine. The organic layers were dried over anhydrous $MgSO_4$ and filtered, and the filtrate evaporated. The residue was purified by silica gel column chromatography (5:5 hexane/EtOAc to 9:1 CH_2Cl_2/MeOH) to afford the desired product **20** as a white crystal (2.67 g, 95%). TLC (SiO_2, 2/8 MeOH/CH_2Cl_2): R_f = 0.25; ^{1}H NMR

(DMSO-d_6, 400 MHz) δ 7.06 (d, J = 6.2 Hz, 1H), 3.92–3.91 (m, 1H), 2.48 (t, J = 5.5 Hz, 2H), 1.99–1.94 (m, 1H), 1.83–1.79 (m, 1H), 1.34 (s, 9H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 173.1, 155.7, 119.9, 78.4, 52.4, 28.2, 28.0, 26.7, 13.7; MS (FAB, m/z) 229 $(M+H)^+$.

4.6.9. *tert*-Butyl-*N*-{(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-cyanopropanoyl}glycinate (21). To a solution of Boc-Asn-OH (3.0 g, 13.3 mmol) in dry pyridine (20 mL) was added DCC (2.89 g, 13.9 mmol) in dry acetone (40 mL) under nitrogen. The suspension was stirred at room temperature for 2 h and the DCC salt was separated from the reaction mixture by filtration. The filtrate was concentrated and $CHCl_3$ was added to the reaction residue, which was then washed repeatedly with 6% $HCl_{(aq)}$ and water. The organic layers were dried over anhydrous Na_2SO_4 and filtered, and the filtrate evaporated. The crude product was crystallized from EtOAc/hexane (1:1) to give (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-cyanopropanoic acid (**19**) as a white crystal (2.1 g, 75%). TLC (SiO_2, 9:1 CH_2Cl_2/MeOH): R_f = 0.1. Compound **21** was synthesized starting from **19** by the similar pathway used to prepare **13**. Dipeptide **21** was obtained as a white crystal (2.6 g, 79%). TLC (SiO_2, 9:1 CH_2Cl_2/MeOH): R_f = 0.6; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.18 (s, 1H), 5.93 (s, 1H), 4.57 (m, 1H), 3.85 (t, J = 4.9 Hz, 2H), 2.83 (d, J = 3.8 Hz, 2H), 1.38 (s, 18H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.1, 168.4, 155.2, 116.9, 82.3, 80.8, 50.4, 42.0, 28.1, 27.8, 21.1; MS (FAB, m/z) 328 $(M+H)^+$; HRFABMS calcd for $C_{15}H_{26}O_5N_3$ 328.1872, found 328.1876.

4.6.10. *tert*-Butyl-5-azanylidyne-*N*-(*tert*-butoxycarbonyl)-L-norvalylglycinate (22). Compound **22** was synthesized from **20** by the similar pathway used to prepare **21**. Dipeptide **22** was obtained as a white crystal (1.76 g, 88%). TLC (3:7 hexane/EtOAc): R_f = 0.65; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.79 (s, 1H), 5.14 (s, 1H), 4.03 (br, 2H), 2.47–2.36 (m, 2H), 2.14–2.13 (m, 1H), 1.91–1.89 (m, 1H), 1.45 (s, 9H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.8, 168.5, 155.6, 119.0, 82.3, 80.5, 41.9, 28.7, 28.2, 28.0, 13.7; MS (FAB, m/z) 342 $(M+H)^+$, HRFABMS calcd for $C_{16}H_{28}O_5N_3$ 342.2029, found 342.2024.

4.6.11. N^5-[(1*S*)-2-[(2-*tert*-Butoxy-2-oxoethyl)amino]-1-(cyanomethyl)-2-oxoethyl]-N^2-(*tert*-butoxycarbonyl)glutamine *tert*-butyl ester (23). Compound **23** was synthesized from **21** by the similar pathway used to prepare **15**. Tripeptide **23** was obtained as a white crystal (1.1 g, 69%). TLC (SiO_2, EtOAc): R_f = 0.75; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.46 (d, J = 6.4 Hz, 1H), 7.34 (d, J = 4.6 Hz, 1H), 5.38 (d, J = 7.1 Hz, 1H), 4.81 (d, J = 6.7 Hz, 1H), 4.17 (s, 1H), 3.90 (dd, J = 5.4, 1.7 Hz, 2H), 2.95 (m, 1H), 2.42–2.36 (m, 4H), 2.18–2.14 (m, 1H), 1.85–1.83 (m, 1H), 1.44 (s, 9H), 1.43 (s, 9H), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.8, 171.3, 168.7, 168.4, 156.0, 117.1, 82.4, 82.3, 80.1, 53.1, 49.6, 42.1, 32.1, 29.0, 28.3, 28.0, 27.9, 20.2; MS (FAB, m/z) 513 $(M+H)^+$; HRFABMS calcd for $C_{24}H_{41}N_4O_8$ 513.2924, found 513.2942.

4.6.12. *tert*-Butyl-*N*-(*tert*-butoxycarbonyl)-α-(*tert*-butyl)-γ-glutamyl-5-azanylidyne-L-norvalylglycinate (24). Compound **24** was synthesized from **22** by the similar pathway used to prepare **23**. Tripeptide **24** was obtained as a white crystal (1.47 g, 45%). TLC (3:7 hexane/EtOAc): R_f = 0.6; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.92 (d, J = 5.1 Hz, 1H), 6.89 (t, J = 3.8 Hz, 1H), 5.27 (d, J = 5.1 Hz, 1H), 4.59 (dd, J = 10.6, 5.6 Hz, 1H), 4.10 (s, 1H), 3.89 (d, J = 4.3 Hz, 2H), 2.54–2.46 (m, 2H), 2.34–2.25 (m, 3H), 2.18–2.16 (m, 1H), 2.07–2.00 (m, 1H), 1.82–1.79 (m, 1H), 1.45 (s, 9H), 1.44 (s, 9H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.6, 171.3, 170.5, 168.4, 155.8, 119.2, 82.2, 82.1, 79.8, 53.3, 51.7, 41.9, 32.1, 28.67, 28.2, 27.9, 27.9, 13.6; MS (FAB, m/z) 527 $(M+H)^+$, HRFABMS calcd for $C_{25}H_{43}O_8N_4$ 527.3074, found 527.3081.

4.7. Synthesis and characterization of bifunctional inhibitors

4.7.1. [(3a*R*,5*S*,7a*R*)-3a,5-Dihydroxy-7a-methyloctahydro-1*H*-inden-1-one]-aspartyl-glutathione (6). To a stirring solution of **40** (105 mg, 0.1 mmol) in anhydrous CH_2Cl_2 (5 mL) was added neat DBU (14 μL, 0.18 mmol). The mixture was stirred at room temperature for 30 min. The resulting solution was then removed on a rotary evaporator and the residue was purified by flash chromatography (5:5 hexane/EtOAc to EtOAc) to afford the free amine compound as a white crystal (74 mg, 95%). TLC (SiO_2, EtOAc): R_f = 0.1. A solution of the free amine (74 mg, 0.1 mmol), TFA (2 mL), and 2% ddH_2O (40 μL) was stirred at 0 °C for 2 h. The TFA solution was then removed on a rotary evaporator and the colorless oil was triturated with ether to give **6** as a white precipitate. The precipitate was dissolved in water/acetonitrile/TFA (70:30:0.1) and the crude product was purified by RP-HPLC (gradient: 100% A, 5 min isocratic, 10 min to 95% A and 5% B, and then in 12 min to 100% B). The product **6** was obtained as a white solid (74%); ^{1}H NMR (D_2O, 400 MHz) δ 5.15–5.14 (m, 1H), 4.78–4.77 (m, 1H), 4.56 (dd, J = 11.5, 4.8 Hz, 1H), 4.51 (t, J = 5.0 Hz, 1H), 4.42 (dd, J = 11.5, 4.3 Hz, 1H), 4.09–4.00 (m, 3H), 3.27 (dd, J = 18.3, 5.8 Hz, 1H), 3.16 (dd, J = 18.3, 4.7 Hz, 1H), 2.67–2.58 (m, 3H), 2.52–2.45 (m, 1H), 2.26–2.06 (m, 5H), 1.87–1.84 (m, 2H), 1.76 (dd, J = 14.5, 8.3 Hz, 1H), 1.46–1.36 (m, 2H), 1.03 (s, 3H); ^{13}C NMR (D_2O, 100 MHz) δ 225.7, 174.7, 173.0, 172.4, 171.1, 170.6, 168.1, 78.7, 73.4, 64.7, 53.1 (d, J = 27.1 Hz), 52.4, 49.2, 41.3, 38.5, 34.2, 33.8, 31.8, 31.1, 26.7, 26.2, 25.6, 16.3; MS (FAB, m/z) 573 $(M+H)^+$; HRFABMS calcd for $C_{24}H_{37}O_{12}N_4$ 573.2408, found 573.2401; Analytical HPLC: Method 6: t^R, 4.04 min, single peak (100% area); Method 4: t^R 10.84 min, single peak (100% area).

4.7.2. Epiandrosterone-aspartyl-glutathione (7). Compound **7** was prepared from **41** by the similar pathway used to prepare **6**. The final crude product was dissolved in water/acetonitrile/TFA (60:40:0.1) and purified by RP-HPLC (gradient: 60% A and 40% B, 25 min to 40% A and 60% B, and then in 5 min to 10% A and 90% B). The product **7** was obtained as a white solid (30%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.82 (t, J = 5.0 Hz, 1H), 4.64 (d, J = 5.6 Hz, 2H), 4.40 (t, J = 5.4 Hz, 1H), 4.03–3.93 (m, 3H), 3.12 (d, J = 5.5 Hz, 2H), 2.66–2.62 (m, 2H), 2.47 (dd, J = 19.1, 8.9 Hz, 1H), 2.25–2.22 (m, 2H), 2.12–2.07 (m, 1H), 1.90–1.77 (m, 5H), 1.70–1.48 (m, 6H), 1.44–1.26 (m, 7H), 1.14–1.10 (m, 2H), 0.94 (s, 3H),

0.91 (s, 3H), 0.82–0.81 (m, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 229.7, 174.5, 172.5, 172.2, 171.0, 170.3, 168.2, 77.8, 64.4, 53.6, 52.9, 52.3, 50.7, 49.0, 48.5, 44.0, 41.0, 36.0, 35.9, 35.1, 34.4, 33.6, 33.0, 30.9, 30.3, 27.8, 26.6, 25.5, 21.3, 20.0, 13.2, 11.4; MS (*m*/*z*) 679 $(M+H)^+$; HRMS-MALDI calcd for $C_{33}H_{51}N_4O_{11}$ $(M+H)^+$, 679.3554, found 679.3553; Analytical HPLC: Method 8: t_R, 8.62 min (98% area); Method 7: t_R 12.44 min (97% area).

4.7.3. Prasterone-aspartyl-glutathione (8). Compound **8** was prepared from **42** by the similar pathway used to prepare **6**. The crude product was dissolved in water/acetonitrile/TFA (70:30:0.1) and purified by RP-HPLC (gradient: 70% A and 30% B, 10 min to 55% A and 45% B, and then in 4 min to 100% B). Tripeptide **8** was obtained as a white solid (48 mg, 48%). ^{1}H NMR (MeOH-d_4, 500 MHz) δ 5.52 (d, J = 5.0 Hz, 1H), 4.77–4.76 (m, 1H), 4.74–4.68 (m, 1H), 4.60 (dd, J = 11.3, 4.9 Hz, 1H), 4.49 (t, J = 5.0 Hz, 1H), 4.40 (dd, J = 11.4, 4.0 Hz, 1 H), 4.07–3.97 (m, 3H), 3.29 (dd, J = 18.1, 5.4 Hz, 1H), 3.14 (dd, J = 18.1, 4.9 Hz, 1H), 2.62 (t, J = 5.9 Hz, 2H), 2.56 (dd, J = 19.4, 8.4 Hz, 1H), 2.39–2.37 (m, 2H), 2.24–1.91 (m, 9H), 1.81–1.53 (m, 7H), 1.41–1.10 (m, 3H), 1.08 (s, 3H), 0.89 (s, 3H); ^{13}C NMR (D_2O, 100 MHz) δ 229.3, 174.7, 172.9, 172.7, 171.1, 170.5, 168.2, 140.4, 122.9, 77.8, 64.7, 53.2, 52.5, 51.2, 49.8, 49.2, 48.4, 48.1, 41.3, 37.2, 36.6, 36.4, 36.1, 33.9, 31.2, 31.1, 30.9, 30.5, 26.9, 25.7, 21.6, 20.0, 18.9, 13.1; MS (FAB, *m*/*z*) 677 $(M+H)^+$; HRFABMS calcd for $C_{33}H_{48}O_{11}N_4Na$ 699.3217, found 699.3217; Analytical HPLC: Method 9: t_R, 5.36 min (93% area); Method 7: t_R 12.07 min, single peak (99% area).

4.7.4. [(3a*R*,5*R*,7a*R*)-3a,5-Dihydroxy-7a-methyloctahydro-1*H*-inden-1-one]-succinyl-glutathione (9a). A mixture of **47a** (76 mg, 0.16 mmol), TFA (3 mL), and 2% ddH_2O (60 μL) was stirred at 0 °C for 2 h. The TFA solution was then removed on a rotary evaporator and the colorless oil was triturated with ether to give a white precipitate. The crude precipitate was dissolved in water/acetonitrile/TFA (70:30:0.1) and purified by RP-HPLC (gradient: 90% A, 20 min to 85% A, then in 5 min to 20% A) to afford **9a** as a white solid (40 mg, 66%). ^{1}H NMR (D_2O, 400 MHz) δ 4.90–4.85 (m, 1H), 4.72 (t, J = 5.8 Hz, 1H), 4.39 (dd, J = 11.3, 4.9 Hz, 1H), 4.34 (dd, J = 11.5, 6.2 Hz, 1H), 4.00 (t, J = 6.4 Hz, 1H), 3.93 (d, J = 2.0 Hz, 2H), 2.65–2.57 (m, 6H), 2.40–2.37 (m, 2H), 2.25–2.08 (m, 4H), 1.86–1.83 (m, 2H), 1.66 (dd, J = 12.5, 11.2 Hz, 1H), 1.53–1.35 (m, 3H), 1.04 (s, 3H); ^{13}C NMR (D_2O, 100 MHz) δ 223.3, 174.7, 173.9, 173.8, 172.7, 171.8, 171.6, 80.3, 72.6, 64.8, 53.9, 53.8, 42.0, 39.6, 34.4, 32.5, 32.4, 30.4, 30.3, 30.0, 27.5, 27.1, 13.9; MS (LCQ, *m*/*z*) 588 $(M+H)^+$; HRFABMS calcd for $C_{24}H_{36}O_{12}N_3$ 558.2299, found 558.2299; Analytical HPLC: Method 10: t_R, 4.23 min, single peak (100% area); Method 4: t_R 9.71 min, single peak (100% area).

4.7.5. [(3a*R*,5*S*,7a*R*)-3a,5-Dihydroxy-7a-methyloctahydro-1*H*-inden-1-one]-succinyl-glutathione (9b). Compound **9b** was prepared from **47b** by the similar pathway used to prepare **9a**. The crude product was purified by RP-HPLC (gradient: 90% A and 10% B, 20 min to 85% A and 15% B, and then in 5 min to 20% A and 80% B) to afford **9b** as a white solid (68%). ^{1}H NMR (MeOH-d_4, 400 MHz) δ 5.03–4.99 (m, 1H), 4.73 (dd, J = 6.0, 4.9 Hz, 1H), 4.40 (dd, J = 11.3, 4.8 Hz, 1H), 4.34 (dd, J = 11.3, 6.2 Hz, 1H), 3.98 (t, J = 6.3 Hz, 1H), 3.94 (d, J = 2.8 Hz, 2H), 2.65–2.57 (m, 6H), 2.53–2.44 (m, 1H), 2.36–2.11 (m, 4H), 2.04–1.95 (m, 2H), 1.93–1.77 (m, 3H), 1.60 (dd. J = 14.1, 8.4 Hz, 1H), 1.38–1.29 (m, 2H), 0.98 (s, 3H); ^{13}C NMR (D_2O, 100 MHz) δ 226.4, 174.6, 174.4, 174.2, 172.9, 172.4, 171.3, 78.8, 70.9, 63.8, 53.3, 52.9, 52.7, 41.3, 38.3, 34.2, 31.9, 31.1, 29.4, 28.9, 26.9, 25.9, 25.7, 15.7; MS (FAB, *m*/*z*) 558 $(M+H)^+$; HRFABMS calcd for $C_{24}H_{36}O_{12}N_3$ 558.2299, found 558.2293; Analytical HPLC: Method 10: t_R, 6.44 min, single peak (98% area); Method 4: t_R 11.31 min, single peak (100% area).

4.7.6. Epiandrosterone-succinyl-glutathione (10). Compound **10** was prepared from **48** by the similar pathway used to prepare **9a**. The crude product was purified by RP-HPLC (gradient: 50% A, 5 min to 40% A, 15 min to 10% A, and then 5 min to 100% B) to afford **10** as a white solid (34%). ^{1}H NMR (MeOH-d_4, 400 MHz) δ 4.67 (t, J = 5.9 Hz, 1H), 4.64–4.60 (m, 1H), 4.34 (dd, J = 11.3, 4.8 Hz, 1H), 4.29 (dd, J = 11.3, 6.2 Hz, 1H), 3.96 (t, J = 6.4 Hz, 1H), 3.88 (d, J = 1.9 Hz, 2H), 2.57–2.54 (m, 6H), 2.38 (dd, J = 19.1, 8.7 Hz, 1H), 2.17–1.88 (m, 4H), 1.79–1.45 (m, 9H), 1.36–1.14 (m, 7H), 1.04–0.98 (m, 2H), 0.85 (s, 3H), 0.82 (s, 3H), 0.75–0.69 (m, 1H); ^{13}C NMR (MeOH-d_4, 100 MHz) δ 224.1, 174.7, 174.0, 173.92, 172.7, 171.8, 171.6, 75.6, 64.8, 55.9, 54.0, 53.8, 52.9, 46.2 42.0, 38.0, 36.9, 36.8, 36.5, 35.2, 33.0, 32.5, 32.1, 30.4, 30.0, 29.7, 28.6, 27.1, 22.8, 21.7, 14.3, 12.7; MS (FAB, *m*/*z*) 664 $(M+H)^+$; HRFABMS calcd for $C_{33}H_{50}O_{11}N_3$ 664.3445, found 664.3445; Analytical HPLC: Method 11: t_R, 4.77 min, single peak (100% area); Method 7: t_R 10.40 min, single peak (100% area).

4.7.7. Prasterone-succinyl-glutathione (11). Compound **11** was prepared from **49** by the similar pathway used to prepare **9a**. The crude product was dissolved in water/acetonitrile/TFA (70:30:0.1) and purified by RP-HPLC (gradient: 50% A, 5 min to 40% A, then in 20 min to 100% B) to afford **11** as a white solid (80 mg, 71%). ^{1}H NMR (MeOH-d_4, 400 MHz) δ 5.43 (d, J = 4.9 Hz, 1H), 4.72 (dd, J = 6.0, 4.9 Hz, 1H), 4.59–4.52 (m, 1H), 4.41–4.32 (m, 2H), 4.01 (t, J = 6.5 Hz, 1H), 3.93 (d, J = 1.9 Hz, 2H), 2.64–2.57 (m, 7H), 2.45 (dd, J = 19.1, 8.4 Hz, 1H), 2.34 (d, J = 7.7 Hz, 2H), 2.22–2.05 (m, 4H), 1.95–1.71 (m, 4H), 1.68–1.53 (m, 6H), 1.36–1.26 (m, 2H), 1.52–1.08 (m, 1H), 1.06 (s, 3H), 1.06–1.04 (m, 1H), 0.89 (s, 3H); ^{13}C NMR (MeOH-d_4, 100 MHz) δ 226.8, 175.1, 174.6, 174.54, 173.4, 171.8, 141.2, 123.3, 76.2, 64.9, 54.2, 53.8, 52.8, 51.5 42.1, 38.9, 38.0, 37.8, 37.0, 32.6, 32.4, 31.7, 30.3, 29.9, 28.6, 27.1, 22.8, 21.3, 19.8, 14.0; MS (FAB, *m*/*z*) 662 $(M+H)^+$; HRFABMS calcd for $C_{33}H_{48}O_{11}N_3$ 662.3289, found 662.3302; Analytical HPLC: Method 11: t_R, 9.39 min, single peak (97% area); Method 7: t_R, 12.26 min (91% area).

4.7.8. (17-Chloro-3β-hydroxy-androsta-5,16-diene)-succinyl-glutathione (12). Compound **12** was prepared from **50** by the similar pathway used to prepare **9a**. The crude

product was purified by RP-HPLC (gradient: 30% A and 70% B, 5 min to 20% A and 80% B, and then in 20 min to 10% A and 90% B) to afford **12** as a white solid (29%). ^{1}H NMR (MeOH-d_4, 500 MHz) δ 5.61–5.61 (m, 1H), 5.39 (d, J = 4.4 Hz, 1H), 4.70 (t, J = 5.9 Hz, 1H), 4.56–4.49 (m, 1H), 4.37 (dd, J = 11.3, 4.8 Hz, 1H), 4.33 (dd, J = 11.3, 6.1 Hz, 2H), 3.95–3.83 (m, 3H), 2.63–2.54 (m, 6H), 2.31 (d, J = 7.8 Hz, 2H), 2.17–2.12 (m, 3H), 1.98–1.76 (m, 6H), 1.71–1.48 (m, 6H), 1.31 (td, J = 12.6, 4.6 Hz, 2H), 1.15–1.09 (m, 1H), 1.07 (s, 3H), 0.89 (s, 3H); ^{13}C NMR (MeOH-d_4, 125 MHz) δ 174.7, 171.9, 173.9, 172.7, 171.9, 171.6, 146.1, 141.6, 125.9, 123.4, 75.90, 64.8, 57.4, 53.9, 52.2, 42.0, 39.3, 38.3, 38.2, 35.1, 32.2, 32.0, 31.6, 31.0, 30.3, 30.0, 28.9, 27.1, 21.8, 19.8, 15.5; MS (FAB, *m*/*z*) 680 M^+; HRFABMS calcd for $C_{33}H_{47}O_{10}N_3Cl$ 680.2905, found 680.2944; Analytical HPLC: Method 12: t_R, 10.65 min, single peak (97% area); Method 7: t_R 13.85 min, single peak (100% area).

4.7.9. 4-[(3a*R*,5*S*,7a*R*)-3a-hydroxy-7a-methyloctahydro-1*H*-inden-1-one-5]-yl-β-*tert*-butyl-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-aspartate (31). To a solution of **27b** (0.44 g, 2.4 mmol), Fmoc-Asp(O-*t*-Bu)-OH (1.1 g, 2.4 mmol), and DMAP (87 mg, 0.7 mmol) in anhydrous CH_2Cl_2 (50 mL) was added DCC (0.59 g, 2.8 mmol) in anhydrous CH_2Cl_2 (1.5 mL) dropwise under nitrogen. The suspension was stirred at room temperature for 3 h and then the DCC salt was separated from the reaction mixture by filtration. The filtrate was concentrated and purified by silica gel column chromatography (hexane/EtOAc 9:1 to 6:4) to afford **31** as a white solid (1.2 g, 84%). TLC (5:5 hexane/EtOAc): R_f = 0.5; mp: 72–74 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.73 (d, J = 7.5 Hz, 2H), 7.56 (d, J = 7.1 Hz, 2H), 7.37 (t, J = 7.4 Hz, 2H), 7.27 (t, J = 7.4 Hz, 2H), 5.82 (d, J = 8.7 Hz, 1H), 5.06–5.05 (m, 1H), 4.53–4.50 (m, 1H), 4.35 (d, J = 7.2 Hz, 2H), 4.20 (t, J = 7.1 Hz, 1H), 2.86 (dd, J = 16.9, 4.5 Hz, 1H), 2.73 (dd, J = 15.9, 3.1 Hz, 1H), 2.48–2.42 (m, 1H), 2.27–2.18 (m, 1H), 2.07–2.01 (m, 1H), 1.96–1.85 (m, 3H), 1.75–1.72 (m, 1H), 1.62–1.56 (m, 1H), 1.41 (s, 9H), 1.37–1.30 (m, 2H), 0.98 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 218.7, 170.2, 169.9, 156.0, 143.7, 143.6, 141.2, 127.7, 127.0, 125.0, 119.9, 81.9, 78.1, 71.1, 67.3, 52.4, 50.6, 47.0, 40.1, 37.6, 34.2, 32.9, 28.0, 27.1, 26.9, 16.6; MS (FAB, *m*/*z*) 578 $(M+H)^+$; HRFABMS calcd for $C_{33}H_{40}O_8N$ 578.2754, found 578, 2750.

4.7.10. (5α-Androstan-17-one-3β)-yl-β-*tert*-butyl-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-aspartate (32). Compound **32** was prepared from **28** by the similar pathway used to prepare **31**. Monopeptide **32** was obtained as a white crystal (58%). TLC (6:4 hexane/EtOAc): R_f = 0.6; mp = 82 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.48 (d, J = 7.5 Hz, 2H), 7.58 (t, J = 6.6 Hz, 2H), 7.37 (t, J = 7.5 Hz, 2H), 7.28 (td, J = 7.4, 1.1 Hz, 2H), 5.82 (d, J = 8.6 Hz, 1H), 4.77–4.74 (m, 1H), 4.54–4.74 (m, 1H), 4.42–4.37 (m, 1H), 4.31–4.28 (m, 1H), 4.22 (t, J = 7.2 Hz, 1H), 2.90 (dd, J = 16.9, 4.8 Hz, 1H), 2.74 (dd, J = 16.9, 4.5 Hz, 1H), 2.40 (q, J = 8.3 Hz, 1H), 2.05–2.00 (m, 1H), 2.00–1.72 (m, 7H), 1.62–1.46 (m, 5H), 1.43 (s, 9H), 1.28–1.19 (m, 7H), 1.01–0.91 (m, 1H), 0.82 (s, 3H), 0.80 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 221.0, 170.3, 169.9, 155.9, 143.8, 143.6, 141.2, 141.1, 127.6, 127.0, 125.1, 125.0, 119.9, 81.6, 76.7, 75.0, 67.1, 54.1, 51.2, 50.6, 47.6, 47.0, 44.4, 37.7, 36.5, 35.7, 35.5, 34.9, 34.8, 33.6, 31.4, 30.6, 28.1, 28.0, 27.9, 27.9, 27.2, 21.7, 20.4, 13.7, 12.1; MS (*m*/*z*) 684 $(M+H)^+$; HRMS-MALDI calcd for $C_{42}H_{54}NO_7$ $(M+H)^+$ 684.3900, found 684.3903.

4.7.11. (5-Androsten-17-one-3β)-yl-β-*tert*-butyl-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-aspartate (33). Compound **33** was prepared from **29** by the similar pathway used to prepare **31**. The product **33** was obtained as a white crystal (1.1 g, 80%). TLC (SiO_2, 6:4 hexane/EtOAc): R_f = 0.6; mp = 84–86 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.73 (d, J = 7.5 Hz, 2H), 7.58 (t, J = 6.6 Hz, 2H), 7.37 (t, J = 7.3 Hz, 2H), 7.28 (td, J = 7.4, 0.9 Hz, 2H), 5.83 (d, J = 8.5 Hz, 1H), 5.37 (d, J = 4.5 Hz, 1 H), 4.69–4.66 (m, 1H), 4.57–4.52 (m, 1 H), 4.40 (dd, J = 10.4, 7.2 Hz, 1H), 4.31 (dd, J = 10.5, 7.4 Hz, 1H), 4.22 (t, J = 7.2 Hz, 1H), 2.92 (dd, J = 16.8, 4.6 Hz, 1H), 2.75 (dd, J = 16.8, 4.4 Hz, 1H), 2.42 (dd, J = 19.3, 8.6 Hz, 1H), 2.33–2.28 (m, 2H), 2.10–2.01 (m, 2H), 1.91–1.80 (m, 4H), 1.64–1.46 (m, 6 H), 1.44 (s, 9H), 1.29–1.21 (m, 2H), 1.11 (dd, J = 14.6, 4.6 Hz, 1H), 1.00–0.95 (m, 1H), 0.99 (s, 3H), 0.85 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 220.8, 170.0, 169.9, 155.9, 143.8, 143.6, 141.2, 139.5, 127.6, 127.0, 125.1, 125.0, 122.1, 119.9, 81.6, 77.2, 75.2, 67.1, 51.5, 51.5, 50.6, 50.0, 47.4, 47.0, 37.7, 36.7, 36.6, 35.7, 31.3, 31.3, 30.6, 28.0, 28.0, 27.9, 27.9, 27.5, 21.8, 20.2, 19.2, 13.4; MS (FAB, *m*/*z*) 682 $(M+H)^+$; HRFABMS calcd for $C_{42}H_{52}O_7N$ 682.3744, found 682.3755.

4.7.12. (5α-Androstan-17-one-3β)-yl-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-aspartate (35). To a solution of **32** (590 mg, 0.86 mmol) in dry CH_2Cl_2 (15 mL) at 0 °C was added TFA (1 mL, 13 mmol). The solution was stirred at 0 °C for 2 h. The resulting mixture was then removed on a rotary evaporator and the residue was partitioned between CH_2Cl_2 and $NaHCO_3$. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The crude residue was purified by chromatography (8:2 hexane/EtOAc to 5:5) to afford **35** as a white crystal (0.5 g, 92%). TLC (6:4 hexane/EtOAc): R_f = 0.15; mp = 103 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.55 (br, 1H), 7.73 (d, J = 7.4 Hz, 2H), 7.58 (d, J = 7.5 Hz, 2H), 7.37 (t, J = 7.4 Hz, 2H), 7.30–7.26 (m, 2H), 5.92 (d, J = 8.4 Hz, 1H), 4.77–4.72 (m, 1H), 4.63–4.59 (m, 1H), 4.47–4.30 (m, 2H), 4.21 (t, J = 7.1 Hz, 1H), 3.06 (dd, J = 17.2, 4.6 Hz, 1H), 3.06 (dd, J = 17.2, 4.6 Hz, 1H), 2.91 (dd, J = 16.8, 4.4 Hz, 1H), 2.40 (q, J = 8.5 Hz, 1H), 2.06–2.01 (m, 1H), 1.83–1.66 (m, 6H), 1.59–1.56 (m, 2H), 1.49–1.43 (m, 3H), 1.35–1.29 (m, 1H), 1.26–1.25 (m, 5H), 0.96–0.87 (m, 2H), 0.81 (s, 3H), 0.78 (s, 3H), 0.63–0.60 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 221.1, 175.1, 170.0, 156.0, 143.6, 143.6, 141.2, 127.7, 127.0, 125.1, 119.9, 75.4, 67.3, 54.1, 51.2, 50.4, 47.7, 47.0, 44.4, 36.5, 35.7, 35.5, 34.8, 33.5, 31.3, 30.6, 28.1, 27.1, 21.6, 20.3, 13.7, 12.1, 12.1; MS (*m*/*z*) 628 $(M+H)^+$; HRMS-MALDI calcd for $C_{38}H_{46}NO_7$ $(M+H)^+$, 628.3274, found 628.3282.

4.7.13. (5-Androsten-17-one-3β)-yl-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-aspartate (36). Compound **36** was prepared from **33** by the similar pathway used to prepare

34. The product was purified by chromatography (hexane/EtOAc 8:2 to 5:5) to afford **36** as a yellow crystal (0.9 g, 89%). TLC (SiO_2, 5:5 hexane/EtOAc): $R_f = 0.5$; mp = 112–126 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.74 (d, $J = 7.5$ Hz, 2H), 7.58 (d, $J = 7.3$ Hz, 2H), 7.38 (t, $J = 7.4$ Hz, 2 H), 7.29 (t, $J = 7.5$ Hz, 2H), 6.25 (br, 1H), 5.81 (d, $J = 8.5$ Hz, 1H), 5.38 (d, $J = 3.9$ Hz, 1H), 4.69–4.61 (m, 2H), 4.41 (t, $J = 7.6$ Hz, 1H), 4.35 (t, $J = 7.2$ Hz, 1H), 4.22 (t, $J = 7.0$ Hz, 1H), 3.08 (dd, $J = 17.7$, 4.6 Hz, 1H), 2.92 (dd, $J = 17.6$, 4.2 Hz, 1H), 2.44 (dd, $J = 19.3$, 8.6 Hz, 1H), 2.31–2.29 (m, 2H), 2.11–2.02 (m, 2H), 1.94–1.80 (m, 4H), 1.61–1.42 (m, 7H), 1.29–1.22 (m, 2H), 1.14–1.08 (m, 1H), 0.92 (s, 3H), 0.85 (s, 3H); MS (FAB, *m*/*z*) 626 $(M+H)^+$; HRFABMS calcd for $C_{38}H_{44}O_7N$ 626.3118, found 626.3125.

4.7.14. [(3a*R*,5*S*,7a*R*)-3a,5-Dihydroxy-7a-methyloctahydro-1*H*-inden-1-one]-aspartyl-glutathione ester (40). TFA (7 mL, 97 mmol) was added dropwise to a stirred solution of compound **31** (1.0 g, 1.6 mmol) in anhydrous CH_2Cl_2 (15 mL) and the mixture was allowed to stir at 0 °C for 2 h. The reaction solution was then concentrated and CH_2Cl_2 was added to the reaction residue, which was then washed repeatedly with $NaHCO_{3(aq)}$ and water. The organic layers were dried over anhydrous Na_2SO_4 and filtered, and the filtrate evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc 8:2 to 5:5) to afford **34** as a white crystal (0.79 g, 88%). TLC (3:7 hexane/EtOAc): $R_f = 0.25$.

To a solution of **34** (380 mg, 0.75 mmol), **15** (400 mg, 0.79 mmol), and DMAP (91 mg, 0.23 mmol) in anhydrous CH_2Cl_2 (15 mL) was added DCC (170 mg, 0.83 mmol) in anhydrous CH_2Cl_2 (1 mL) under nitrogen. The suspension was stirred at room temperature for 4 h and the DCC salt was separated from the reaction mixture by filtration. The filtrate was concentrated and purified by silica gel column chromatography (hexane/EtOAc 9:1 to 6:4) to afford **40** as a white crystal (0.39 g, 51%). TLC (4:6 hexane/EtOAc): $R_f = 0.25$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.75 (d, $J = 7.5$ Hz, 2H), 7.68 (d, $J = 4.9$ Hz, 2H), 7.38 (t, $J = 7.4$ Hz, 2H), 7.29 (t, $J = 7.4$ Hz, 2H), 7.17 (m, 1H), 6.96 (d, $J = 9.3$ Hz, 1H), 5.36 (d, $J = 8.4$ Hz, 1H), 5.04 (s, 1H), 5.02 (m, 1H), 4.87 (d, $J = 7.4$ Hz, 1H), 4.77–4.69 (m, 2H), 4.36 (d, $J = 5.5$ Hz, 2H), 4.26 (t, $J = 7.3$ Hz, 1H), 4.19 (d, $J = 4.2$ Hz, 1H), 4.11 (dd, $J = 0.9$, 7.2 Hz, 1H), 3.95 (d, $J = 4.7$ Hz, 2H), 3.08 (dd, $J = 16.5$, 4.3 Hz, 1H), 2.86 (dd, $J = 16.5$, 3.4 Hz, 1H), 2.57–2.46 (m, 4H), 2.29–2.22 (m, 3H), 2.12–1.68 (m, 6H), 1.45 (s, 9H), 1.40 (s, 18H), 1.32–1.24 (m, 1H), 1.02 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 218.5, 172.5, 171.5, 169.4, 168.9, 168.3, 156.4, 155.7, 143.8, 143.7, 141.1, 127.6, 127.0, 126.9, 125.2, 125.1, 119.9, 82.4, 80.0, 77.8, 77.2, 72.1, 67.3, 64.7, 53.4, 52.5, 51.0, 50.5, 42.1, 41.0, 38.5, 37.7, 34.3, 32.7, 32.1, 28.8, 28.2, 28.0, 27.9, 26.9, 18.1; MS (FAB, *m*/*z*) 1029 $(M+Na)^+$; HRFABMS calcd for $C_{52}H_{70}O_{16}N_4Na$ 1029.4685, found 1029.4691.

4.7.15. Epiandrosterone-aspartyl-glutathione ester (41). Compound **41** was prepared from **35** by the similar pathway used to prepare **40**. Tripeptide **41** was obtained as a white crystal (40%). TLC (6:4 hexane/EtOAc): $R_f = 0.35$; mp = 112 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.72 (d, $J = 7.5$ Hz, 2H), 7.62 (d, $J = 6.8$ Hz, 2H), 7.36 (t, $J = 7.3$ Hz, 2H), 7.27 (t, $J = 7.4$ Hz, 2H), 7.14 (br, 1H), 6.98 (br, 1H), 6.42 (br, 1H), 5.22 (d, $J = 7.3$ Hz, 1H), 4.79–4.58 (m, 3H), 4.39–4.28 (m, 2H), 4.23–4.07 (m, 3H), 3.90 (t, $J = 5.3$ Hz, 2H), 3.47–3.42 (m, 1H), 3.00–2.85 (m, 2H), 2.43–2.33 (m, 3H), 2.10–2.00 (m, 2H), 1.91–1.87 (m, 3H), 1.77–1.73 (m, 2H), 1.69–1.63 (m, 2H), 1.54–1.49 (m, 3H), 1.39 (m, 27H), 1.35–1.96 (m, 7H), 0.81 (s, 3H), 0.80 (s, 3H), 0.69–0.68 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 221.2, 172.5, 171.4, 170.8, 169.5, 169.1, 168.5, 156.3, 155.7, 143.8, 143.7, 141.2, 127.7, 127.1, 125.2, 119.9, 82.4, 82.2, 79.8, 75.5, 67.3, 64.4, 60.3, 54.1, 53.5, 51.6, 51.3, 50.6, 47.7, 47.0, 44.5, 42.1, 38.6, 37.5, 36.5, 35.8, 35.6, 35.0, 33.8, 32.1, 31.4, 30.7, 28.6, 28.3, 28.2, 27.9, 27.2, 21.7, 20.4, 13.8, 12.2; MS (*m*/*z*) 1113 $(M+H)^+$; HRMS-MALDI calcd for $C_{61}H_{85}N_4O_{15}$ $(M+H)^+$, 1113.6011, found 1113.6014.

4.7.16. Prasterone-aspartyl-glutathione ester (42). Compound **42** was prepared from **36** by the similar pathway used to prepare **40**. Compound **42** was obtained as a white crystal (0.44 mg, 80%). TLC (SiO_2, 6:4 hexane/EtOAc): $R_f = 0.3$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.74 (d, $J = 7.5$ Hz, 2 H), 7.62 (d, $J = 6.8$ Hz, 2H), 7.37 (t, $J = 7.3$ Hz, 2H), 7.28 (tt, $J = 7.5$, 1.4 Hz, 2H), 7.14 (s, 1H), 6.90 (s, 1H), 6.39 (d, $J = 8.0$ Hz, 1H), 5.37 (d, $J = 4.6$ Hz, 1H), 5.21 (d, $J = 5.6$ Hz, 1H), 4.78–4.76 (m, 1H), 4.67–4.60 (m, 2H), 4.40–4.30 (m, 1H), 4.22 (t, $J = 7.2$ Hz, 2H), 4.10 (s, 1H), 3.95 (dd, $J = 18.2$, 5.4 Hz, 1H), 3.87 (dd, $J = 18.2$, 5.1 Hz, 1H), 3.00 (dd, $J = 16.5$, 4.8 Hz, 1H), 2.88 (dd, $J = 16.6$, 3.6 Hz, 1H), 2.44 (dd, $J = 19.3$, 8.6 Hz, 1 H), 2.38–2.27 (m, 3H), 2.11–2.01 (m, 3H), 1.95–1.81 (m, 7H), 1.66–1.44 (m, 5H), 1.41 (s, 9H), 1.39 (s, 9H), 1.39 (s, 9H), 1.30–1.23 (m, 2H), 1.17–1.08 (m, 1H), 1.05–1.01 (m, 4H), 0.86 (s, 3H); ^{13}C NMR($CDCl_3$, 100 MHz) δ 221.0, 172.6, 171.3, 170.7, 169.5, 169.1, 168.2, 156.3, 155.6, 143.8, 143.7, 141.2, 139.3, 127.7, 127.1, 125.2, 122.3, 119.9, 82.4, 82.2, 79.9, 77.2, 75.6, 67.3, 64.4, 51.7, 51.6, 50.6, 50.0, 49.1, 47.5, 47.0, 42.1, 37.9, 37.5, 36.7, 36.6, 32.0, 31.4, 31.3, 30.7, 29.6, 28.6, 28.3, 27.9, 27.9, 27.5, 21.8, 20.3,19.3, 13.5; MS (FAB, *m*/*z*) 1111 $(M+H)^+$, HRFABMS calcd for $C_{61}H_{83}O_{15}N_4$ 1111.5855, found 1111.5849.

4.7.17. 4-[(3a*R*,5*R*,7a*R*)-3a-Hydroxy-7a-methyloctahydro-1*H*-inden-1-one-5]-oxy-4-oxobutanoic acid (43a). To a solution of **27a** (176 mg, 1.0 mmol) and succinic anhydride (0.3 g, 3.0 mmol) in anhydrous pyridine (7 mL) at room temperature was added DMAP (86 mg, 1.0 mmol). The resulting solution was refluxed at 60 °C and the reaction progress was monitored by TLC. After 12 h, the suspension was partitioned between H_2O (40 mL) and EtOAc (60 mL). The organic layer was washed with 6% HCl (3×10 mL), $NaHCO_3$ (2×10 mL), and brine. Drying over $MgSO_4$ and evaporation of the organic layer afforded a residue, which was purified by silica gel column chromatography (hexane/EtOAc 8:2 to 5:5). The product **43a** was obtained as a colorless liquid (0.3 g, 86%). TLC (EtOAc): $R_f = 0.45$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.93–4.85 (m, 1H), 2.59 (s, 4H), 2.41–2.38 (m, 2H), 2.30–2.24 (m, 1H), 2.14–2.09 (m, 1H), 1.89–1.82 (m, 2H), 1.67 (t, $J = 11.2$ Hz, 1H), 1.53–1.33

(m, 3H), 1.05 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 223.7, 176.5, 174.1, 80.6, 72.7, 54.2, 39.9, 34.7, 32.6, 30.8, 30.7, 30.3, 27.7, 14.2; MS (FAB, *m/z*) 285 $(M+H)^+$; HRFABMS calcd for $C_{14}H_{21}O_6$ 285.1338, found 285.1336.

4.7.18. 4-[(3a*R*,5*S*,7a*R*)-3a-Hydroxy-7a-methyloctahydro-1*H*-inden-1-one-5]-oxy-4-oxobutanoic acid (43b). Compound **43b** was prepared from **27b** by the similar pathway used to prepare **43a**. Compound **43b** was obtained as a yellow liquid (85%). TLC (EtOAc): $R_f = 0.45$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.89–4.87 (m, 1H), 2.55–2.46 (m, 4H), 2.40–2.36 (m, 1H), 2.22–2.15 (m, 1H), 2.03–1.98 (m, 1H), 1.93–1.85 (m, 2H), 1.77–1.73 (m, 1H), 1.63–1.61 (m, 1H), 1.47 (dd, $J = 14.2$, 8.4 Hz, 1H), 1.28–1.17 (m, 2 H), 0.87 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 219.6, 177.2, 171.8, 78.3, 70.1, 52.5, 51.9, 39.9, 32.7, 29.2, 28.9, 28.8, 27.0, 16.7; MS (FAB, *m/z*) 285 $(M+H)^+$; HRFABMS calcd for $C_{14}H_{21}O_6$ 285.1338, found 285.1331.

4.7.19. 4-(Epiandrosterone-3β)-oxy-4-oxobutanoic acid (44). Compound **44** was prepared from **28** by the similar pathway used to prepare **43**. Compound **44** was obtained as a white solid (80%). TLC (4:6 hexane/EtOAc): $R_f = 0.45$; mp = 236 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.68 (m, 1H), 2.64–2.54 (m, 4H), 2.40 (q, $J = 8.1$ Hz, 1H), 2.08–1.98 (m, 1H), 1.89–1.87 (m, 1H), 1.77–1.69 (m, 4H), 1.61–1.56 (m, 1H), 1.52–1.42 (m, 3H), 1.35–1.18 (m, 7H), 1.03–0.89 (m, 2H), 0.82 (s, 3H), 0.81 (s, 3H), 0.71–0.64 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 221.4, 177.6, 171.6, 74.0, 54.3, 51.3, 47.8, 44.6, 36.6, 35.8, 35.6, 35.0, 33.8, 31.5, 30.8, 29.2, 29.0, 28.2, 27.3. 21.7, 20.4, 13.8, 12.2; MS (FAB, *m/z*) 391 $(M+H)^+$; HRMS-MALDI calcd for $C_{23}H_{35}O_5$ $(M+H)^+$, 391.2484, found 391.2483.

4.7.20. 4-(5-Androsten-17-one-3β)-oxy-4-oxobutanoic acid (45). Compound **45** was prepared from **29** by the similar pathway used to prepare **43a**. The product **45** was obtained as a white crystal (0.81 g, 61%). TLC (SiO_2, 5:5 hexane/EtOAc): $R_f = 0.50$; mp = 232–234 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.38 (d, $J = 4.9$ Hz, 1 H), 4.65–4.57 (m, 1H), 2.65 (t, $J = 6.1$ Hz, 2H), 2.58 (t, $J = 6.8$ Hz, 2H), 2.44 (dd, $J = 19.4$, 8.5 Hz, 1H), 2.33–2.30 (m, 2H), 2.12–2.02 (m, 2H), 1.96–1.87 (m, 1H), 1.87–1.80 (m, 3H), 1.67–1.59 (m, 3H), 1.57–1.48 (m, 2H), 1.48–1.41 (m, 1H), 1.30–1.22 (m, 2H), 1.15–1.08 (m, 1H), 1.02–0.97 (m, 4H), 0.86 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 221.2, 171.9, 171.5, 139.8, 122.0, 74.3, 51.7, 50.1, 47.5, 37.9, 36.9, 36.7, 35.8, 31.4, 31.3, 30.8, 29.2, 28.8, 27.6, 21.9, 20.3, 19.3, 13.5; MS (FAB, *m/z*) 389 $(M+H)^+$; HRFABMS calcd for $C_{23}H_{33}O_5$ 389.2328, found 389.2332.

4.7.21. 4-(17-Chloro-androsta-5,16-diene-3β)-oxy-4-oxobutanoic acid (46). Compound **46** was prepared from **30** by the similar pathway used to prepare **43a**. Compound **46** was obtained as a white solid (76%). TLC (4:6 hexane/EtOAc): $R_f = 0.45$; mp = 160–161 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.60 (dd, $J = 3.1$, 1.7 Hz, 1H), 5.37 (d, $J = 5.1$ Hz, 1H), 4.65–4.58 (m, 1H), 2.65 (td, $J = 7.5$, 1.7 Hz, 2H), 2.58 (td, $J = 7.1$, 1.3 Hz, 2H), 2.32–2.29 (m, 2H), 2.12–2.29 (m, 1H), 1.95–1.82 (m, 5H), 1.67–1.56 (m, 4H), 1.53–1.46 (m, 2H), 1.32 (td, $J = 12.5$, 4.6 Hz, 1H), 1.15–1.11 (m, 1H), 1.03–1.01 (m, 4H), 0.87 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.9, 171.5, 144.6, 140.0, 124.6, 122.1, 74.4, 55.7, 50.3, 47.4, 38.0, 36.8, 33.6, 31.0, 30.6, 30.5, 29.2, 28.9, 27.6, 20.5, 19.2, 14.9; MS (FAB, *m/z*) 389 $(M+H-H_2O)^+$, HRFABMS calcd for $C_{23}H_{32}O_4Cl$ 407.1989, found 407.1989.

4.7.22. [(3a*R*,5*R*,7a*R*)-3a,5-Dihydroxy-7a-methyloctahydro-1*H*-inden-1-one]-succinyl-glutathione ester (47a). To a solution of **43a** (255 mg, 0.9 mmol), **15** (315 mg, 0.63 mmol), and DMAP (37 mg, 0.30 mmol) in anhydrous CH_2Cl_2 (5 mL) was added DCC (264 mg, 1.0 mmol) in anhydrous CH_2Cl_2 (1 mL) under nitrogen. The suspension was stirred at room temperature for 3 h and the DCC salt was separated from the reaction mixture by filtration. The filtrate was concentrated and purified by silica gel column chromatography (hexane/EtOAc 9:1 to 6:4) to afford **47a** as a white crystal (568 mg, 82%). TLC (3:7 hexane/EtOAc): $R_f = 0.45$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.97–6.95 (m, 2H), 5.26 (s, 1H), 4.93–4.89 (m, 1H), 4.74–4.70 (m, 1H), 4.42 (dd, $J = 11.3$, 5.4 Hz, 1H), 4.38 (dd, $J = 11.3$, 5.1 Hz, 1H), 4.09 (s, 1H), 3.93 (dd, $J = 18.2$, 5.4 Hz, 1H), 3.85 (dd, $J = 18.2$, 5.1 Hz, 1 H), 2.83–2.82 (m, 1H), 2.64–2.56 (m, 4H), 2.55–2.41 (m, 2H), 2.35–2.25 (m, 3H), 2.12–2.05 (m, 2H), 2.00–1.89 (m, 3H), 1.77–1.68 (m, 2H), 1.58–1.46 (m, 1H), 1.43–1.42 (m, 18H), 1 .39 (s, 9H), 1.27–1.22 (m, 1H), 1.05 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 219.7, 172.7, 172.1, 171.7, 171.3, 168.9, 168.5, 155.8, 82.4, 80.1, 78.6, 71.1, 63.6, 53.4, 52.4, 52.2, 42.1, 38.7, 33.5, 32.3, 31.5, 29.4, 29.1, 28.3, 28.0, 27.9, 27.6, 26.2, 14.6; MS (FAB, *m/z*) 770 $(M+H)^+$; HRFABMS calcd for $C_{37}H_{60}O_{14}N_3$ 770.4075, found 770.4075.

4.7.23. [(3a*R*,5*S*,7a*R*)-3a,5-Dihydroxy-7a-methyloctahydro-1*H*-inden-1-one]-succinyl-glutathione ester (47b). Compound **47b** was prepared from **43b** by the similar pathway used to prepare **47a**. The product **47b** was obtained as a white crystal (0.53 mg, 82%). TLC (SiO_2, 3:7 hexane/EtOAc): $R_f = 0.45$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.24 (s, 1 H), 6.99 (d, $J = 6.7$ Hz, 1H), 5.34 (s, 1H), 5.04–4.98 (m, 1 H), 4.73–4.68 (m, 1H), 4.45 (dd, $J = 11.3$, 4.8 Hz, 1H), 4.39 (dd, $J = 11.3$, 5.3 Hz, 1H), 4.14 (s, 1H), 3.95 (dd, $J = 18.2$, 5.4 Hz, 1H), 3.86 (dd, $J = 18.2$, 5.0 Hz, 1H), 2.58–2.53 (m, 5H), 2.53–2.45 (m, 1H), 2.39–2.34 (m, 2H), 2.25–2.06 (m, 2H), 2.05–1.91 (m, 6H), 1.76–1.72 (m, 1H), 1.43–1.42 (m, 18H), 1.40 (s, 9H), 1.31–1.24 (m, 1H), 0.97 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 218.9, 172.9, 172.6, 172.0, 171.53, 169.1, 168.6, 155.9, 82.3, 80.0, 78.0, 70.5, 63.2, 53.5, 52.5, 52.2, 42.1, 40.7, 34.3, 32.8, 32.2, 29.7, 29.3, 28.7, 28.3, 28.0, 27.8, 27.1, 17.5; MS (FAB, *m/z*) 770 $(M+H)^+$; HRFABMS calcd for $C_{37}H_{60}O_{14}N_3$ 770.4075, found 770.4078.

4.7.24. Epiandrosterone-succinyl-glutathione ester (48). Compound **48** was prepared from **44** by the similar pathway used to prepare **47a**. Compound **48** was obtained as a white crystal (73%). TLC (6:4 hexane/EAtOAc): $R_f = 0.3$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.95–6.93 (m, 2H), 5.24 (m, 1H), 4.73–4.63 (m, 2H), 4.42–4.33 (m, 2H), 4.08–4.05 (m, 1H), 3.93–3.81 (m, 2H),

2.57–2.54 (m, 5H), 2.36–2.28 (m, 3H), 2.27–1.86 (m, 3H), 1.74–1.46 (m, 9H), 1.39 (s, 9H), 1.38 (s, 9H), 1.37 (s, 9H), 1.32–0.82 (m, 12H), 0.79 (s, 3H), 0.78 (s, 3H), 0.66–0.64 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 221.2, 172.6, 171.4, 169.0, 168.6, 155.8, 82.3, 79.9, 74.2, 63.6, 54.3, 53.5, 52.3, 51.4, 47.7, 44.7, 42.2, 36.7, 35.9, 35.7, 35.1, 33.9, 33.8, 32.3, 31.6, 30.9, 29.6, 29.2, 28.9, 28.4, 28.0, 27.4, 27.3, 25.6, 24.9, 21.8, 20.5, 13.8, 12.2; MS (FAB, *m*/*z*) 876 $(M+H)^+$; HRFABMS calcd for $C_{46}H_{74}O_{13}N_3$ 876.5222, found 876.5225.

4.7.25. Prasterone-succinyl-glutathione ester (49). Compound **49** was prepared from **45** by the similar pathway used to prepare **47a**. The product **49** was obtained as a white crystal (0.47 g, 81%). TLC (SiO_2, 6:4 hexane/EtOAc): $R_f = 0.25$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.01 (d, $J = 6.5$ Hz, 1H), 6.95 (t, $J = 5.2$ Hz, 1H), 5.37 (d, $J = 4.7$ Hz, 1 H), 5.26 (d, $J = 5.6$ Hz, 1H), 4.75–4.70 (m, 1H), 4.62–4.55 (m, 1H), 4.44 (dd, $J = 11.3$, 4.1 Hz, 1H), 4.36 (dd, $J = 11.3$, 5.0 Hz, 1H), 4.11 (s, 1 H), 3.94 (dd, $J = 18.1$, 5.5 Hz, 1H), 3.85 (dd, $J = 18.1$, 5.1 Hz, 1H), 2.44 (s, 4H), 2.43 (dd, $J = 19.3$, 8.7 Hz, 1H), 2.35–2.29 (m, 3H), 2.14–2.01 (m, 3H), 1.95–1.79 (m, 5H), 1.65–1.46 (m, 7H), 1.43–1.42 (m, 18H), 1.40 (s, 9H), 1.29–1.22 (m, 2H), 1.15–1.11 (m, 1H), 1.01–0.88 (m, 4H), 0.85 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 221.1, 172.7, 172.2, 172.0, 171.3, 169.0, 168,6, 139.8, 122.0, 121.9, 82.3, 79.9, 77.2, 74.3, 74.1, 63.5, 53.3, 52.2, 51.7, 50.3, 50.1, 47.5, 42.0, 38.0, 36.8, 36.7, 35.8, 32.2, 31.4, 31.3, 30.7, 29.4, 29.1, 28.3, 28.0, 27.9, 27.8, 27.6, 21.8, 20.3, 19.3, 13.5; MS (FAB, *m*/*z*) 874 $(M+H)^+$; HRFABMS calcd for $C_{46}H_{72}O_{13}N_3$ 874.5065, found 874.5064.

4.7.26. (17-Chloro-3β-hydroxy-androsta-5,16-diene)-succinyl-glutathione ester (50). Compound **50** was prepared from **46** by the similar pathway used to prepare **47a**. Compound **50** was obtained as a white solid (81%). TLC (6:4 hexane/EtOAc): $R_f = 0.25$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.99–6.93 (m, 2H), 5.59 (t, $J = 1.4$ Hz, 1H), 5.35 (d, $J = 5.0$ Hz, 1H), 5.27 (d, $J = 6.0$ Hz, 1H), 4.73 (dd, $J = 12.5$, 5.2 Hz, 1H), 4.61–4.53 (m, 1H), 4.44 (dd, $J = 11.2$, 5.2 Hz, 1H), 4.36 (dd, $J = 11.2$, 5.0 Hz, 1H), 4.11 (s, 1H), 3.94 (dd, $J = 18.2$, 5.3 Hz, 1H), 3.85 (dd, $J = 18.2$, 4.8 Hz, 1H), 2.58 (s, 4H), 2.34–2.27 (m, 4H), 2.14–2.08 (m, 3H), 1.95–1.73 (m, 5H), 1.66–1.44 (m, 6H), 1.42 (s, 18H), 1.39 (s, 9 H), 1.33–1.26 (m, 1H), 1.11–1.04 (m, 1H), 1.01–0.99 (m, 4H), 0.85 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.5, 172.1, 171.9, 171.3, 168.9, 168.5, 155.7, 144.6, 139.9, 124.5, 122.1, 82.2, 79.9, 74.4, 63.5, 55.6, 53.3, 52.1, 50.3, 47.4, 42.0, 38.0, 36.7, 33.5, 32.2, 30.9, 30.5, 30.4, 29.4, 29.1, 28.9, 28.3, 28.0, 27.9, 27.6, 20.4, 19.1, 14.8; MS (FAB, *m*/*z*) 892 $(M+H)^+$; HRFABMS calcd for $C_{46}H_{71}O_{12}ClN_3$ 892.4727, found 892.4734.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.07.077.

References and notes

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