Novel Analogues of Istaroxime, a Potent Inhibitor of Na^+,K^+-ATPase: Synthesis and Structure−Activity Relationship†

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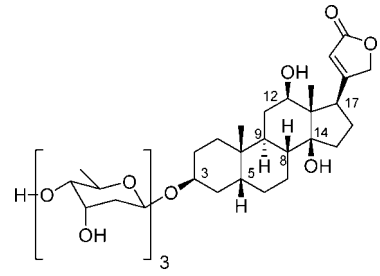
Received March 10, 2008

We report the synthesis and biological properties of novel inhibitors of the Na^+,K^+-ATPase as positive inotropic compounds. Following our previously described model from which Istaroxime was generated, the 5α,14α-androstane skeleton was used as a scaffold to study the space around the basic chain of our lead compound. Some compounds demonstrated higher potencies than Istaroxime on the receptor and the (*E*)-3-[(*R*)-3-pyrrolidinyl]oxime derivative, **15**, was the most potent; as further confirmation of our model, the *E* isomers of the oxime are more potent than the *Z* form. The compounds tested in the guinea pig model induced positive inotropic effects, which are correlated to the in vitro inhibitory potency on the Na^+,K^+-ATPase. The finding that all tested compounds resulted less proarrhythmogenic than digoxin, a currently clinically used positive inotropic agent, suggests that this could be a feature of the 3-aminoalkyloxime derivative class of 5α,14α-androstane.

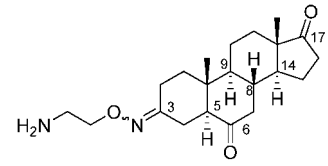
Introduction

The digitalis cardiac glycoside digoxin (Chart 1) is currently one of the most prescribed drugs for the treatment of congestive heart failure (CHF)[a] due to its capability to increase the contractile force of the cardiac muscle, known as positive inotropic effect. The action of digitalis compounds, which improve the pumping performance of the heart, is mainly due to inhibition of the Na^+,K^+-ATPase. This is a membrane protein that promotes the outward transport of Na^+ and the inward transport of K^+ against their concentration gradients. Cardiac glycosides reversibly bind to the extracellular side of the Na^+,K^+-ATPase, thus blocking ATP hydrolysis and ion transport. When the pump is inhibited, Na^+ concentration inside the cell is increased and, as a consequence, Ca^{2+} is introduced by an exchange with Na^+, through the Na^+,Ca^{2+} exchanger. The final result is the availability of higher Ca^{2+} concentrations needed to activate contraction of the myocardium.[1] Digoxin can alleviate symptoms of CHF, improve exercise tolerance, and reduce hospitalization, while having a neutral effect on mortality.[2] A risk related to therapy with digitalis drugs is their proarrhythmogenicity; evidence of digitalis toxicity emerges at a two to three times higher serum concentration than the therapeutic, and disturbances of conduction and cardiac arrhythmia are characteristics of digitalis toxicity.[3]

Chart 1. Structures of Digoxin and Istaroxime



Digoxin



Istaroxime

The search for safer agents prompted a great deal of work.[4] Recently, our group reported a model derived from superposition of cassaine and digitoxigenin[5] and the design of a new series of compounds from a planar steroidal skeleton[6] instead of the bent skeleton typical of digitalis compounds (Figure 1). The alkaloid cassaine, which was first isolated from the bark of *Erythrophleum guineense* in 1935,[7] shares many of the pharmacologic actions of the cardiac glycosides but lacks the structural characteristic typical of cardiac glycosides. By introducing an *O*-(ω-aminoalkyl)oxime chain in position 3 and oxo or hydroxy groups at positions 6 and 17 of the androstane skeleton, we obtained compounds with inhibitory potency on the Na^+,K^+-ATPase comparable to that of digoxin and positive inotropic activity. Most importantly, some of those compounds displayed a higher safety index than digoxin. From this effort, Istaroxime (referred to as PST 2744; Chart 1), now in phase II

† This paper is dedicated to the memory of Michele Giubileo

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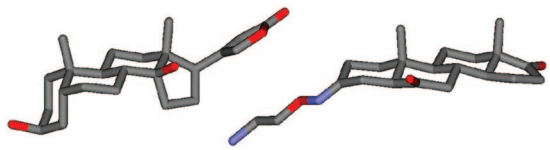
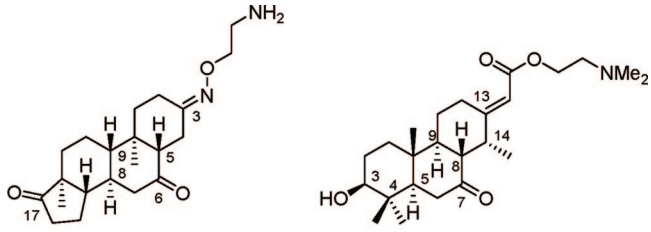
[a] Abbreviations: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; CHF, congestive heart failure; DIPEA, *N*,*N*-diisopropylethylamine; ED_{80}, effective dose (80%); Fmoc, 9-fluorenylmethoxycarbonyl; IC_{50}, inhibitory concentration (50%); rms, root-mean-square; SAR, structure−activity relationship; SERCA2a sarco/endoplasmic reticulum calcium ATPase isoform 2a; TBAF, tetra-*n*-butylammonium fluoride; TEA, triethylamine; THF, tetrahydrofuran.

10.1021/jm800257s CCC: $40.75
Published on Web 07/19/2008

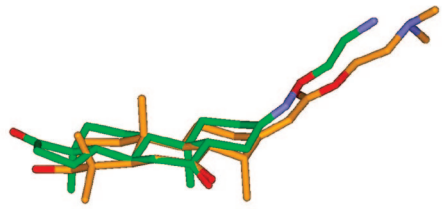


Digitoxigenin and **2** (*E* isomer of Istaroxime)

Figure 1. A 3D model of digitoxigenin, a typical digitalis compound, and compound **2**, the *E* isomer of Istaroxime, showing the different shapes between a bent and planar steroidal skeleton.



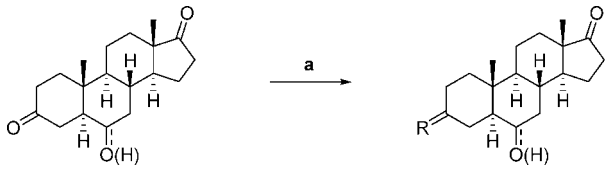
Rotated Istaroxime *E* isomer (**2**) Cassaine



Istaroxime *E* isomer (**2**), green; cassaine, orange

Figure 2. Model of the superposition between cassaine (orange) and **2**, the *E* isomer of Istaroxime (green).

Scheme 1[a]



1a-c **Compounds of Table 1**

[a] Reagents and conditions. **a**: hydroxylamines, THF/water, room temperature. **1a** 6-oxo. **1b** 6α-OH: OH directed downwards. **1c** 6*β*-OH: OH directed upwards.

clinical trials,[8] emerged as a very promising positive inotropic compound that may represent an innovative alternative in the treatment of CHF. The rotated *E* isomer of the most potent compound of that series (**2**) can be well superposed on cassaine, thus confirming our model predicting that the optimal superposition between the basic chains of cassaine and our compounds was expected to be reached with the *E* isomers of the oxime group, as Figure 2 shows.

In the present paper, we report novel Istaroxime analogues having modified oximic chains, with the aim of studying the space around the basic group to obtain compounds with a higher potency and better ratio between the active and toxic doses than the parent compound.

Chemistry

The oximes listed in Table 1 (with the exception of compound **8**), were synthesized from the corresponding ketones of formula **1a**−**c** and the appropriate *O*-substituted hydroxylamine dihydrochlorides (see Supporting Information for the syntheses of the latter compounds) in a THF/water solution at room temperature (Scheme 1, see Experimental Section). The very good regioselectivity of this reaction was confirmed[6] because the oxime at the 3 position was obtained with only traces of dioxime derivatives even though in the presence of two or three keto groups. Almost all the oximes obtained in the coupling reactions were mixtures of *E* and *Z* isomers and, in most cases, not resolved in the two components when their separation proved to be impossible by crystallization or flash chromatography, as reported in our previous papers. The required starting ketones of formula **1a**−**c** (Scheme 1) were prepared as previously reported.[6]

The separation of the *E* and *Z* diastereoisomers of Istaroxime was carried out (Scheme 2) by protection of the amino group as Fmoc derivative. Istaroxime was reacted with FmocCl in the presence of TEA in CH_2Cl_2 to give, after separation by flash chromatography, **27** (*E* isomer, 40% yield) and **28** (*Z* isomer, 40% yield). Each separated isomer was deprotected with TBAF in THF to give the *E* (**2**, 80% yield) and *Z* (**3**, 83% yield) isomers as pure compounds.

Only compound **14** was separated by crystallization in the two *E* and *Z* isomers, **15** and **16**, respectively, while compounds **18**, **21**, and **22** were isolated as *E* isomers only. All remaining oximes were used in the pharmacological tests as mixtures.

Compound **8** was prepared from Istaroxime by reaction with 3,5-dimethyl-1-pyrazolylformamidinium nitrate (Scheme 3).

Results and Discussion

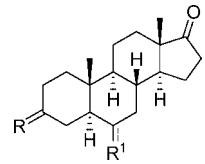
All compounds were tested in vitro for their inhibitory activity on purified dog kidney Na^+,K^+-ATPase, as measured by the ^{32}P-ATP hydrolysis method (see data in Table 1).[9,10] Some compounds, showing high inhibitory potency in vitro, were investigated in vivo for their inotropic activity and lethal effect by slow intravenous infusion in anesthetized guinea pigs (results reported in Table 2). Digoxin was chosen as reference compound because it is the most commonly prescribed cardiac glycoside in the treatment of CHF, while Istaroxime is the lead compound in this series of androstane derivatives.

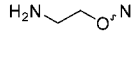
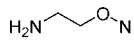
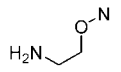
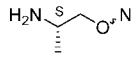
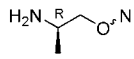
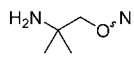
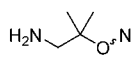
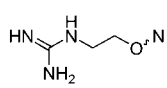
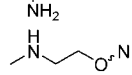
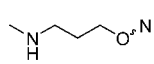
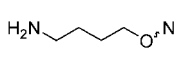
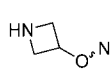
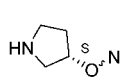
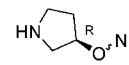
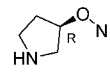
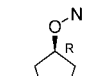
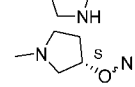
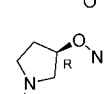
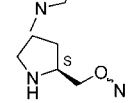
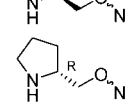
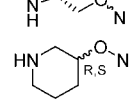
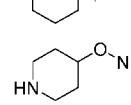
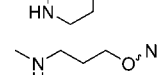
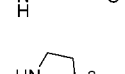
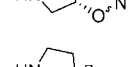
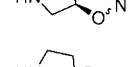
The following Structure−Activity Relationships (SAR) are based on the in vitro data shown in Table 1.

The Diastereoisomers of Istaroxime. One of the most interesting features in our previously reported model[6] focused on cassaine was that the *E*, as opposed to the *Z*, isomers of the oxime were predicted to be more active; some examples supporting this prediction were already reported in that paper, but the separated isomers of Istaroxime were not disclosed. *E* isomer **2** was now found to be 11 times more potent on the Na^+,K^+-ATPase than *Z* isomer **3**, thus further confirming the prediction and the model.

Modified Chains with Primary Amines. The introduction of a methyl group in α position to the amine, both in the *S* (**4**) and *R* (**5**) configuration, gave compounds with equal activity and a three times lower potency than Istaroxime. Two methyl groups in this position of the chain (**6**) reduced potency 260 times, when compared to our lead compound, and more than 70 times when compared with mono-methyl derivatives. Surprisingly, two methyl groups in *β* position to the amino group gave compound **7**, which was five times more potent than Istaroxime. The elongation of an alkylenic spacer between the oximic group and the basic head gave compound **11**, which was 16 times less potent than Istaroxime. Taking the latter result into account, as well as that of the previously reported compound with a $(CH_2)_3$ alkylenic chain,[6] about four times less active than

Table 1. Structure and Na^+,K^+-ATPase Inhibition for Compounds **2**−**26**



Compound	*E,Z*	R	R^1	Na^+,K^+-ATPase Inhibition, IC_{50},[a] μM
Digoxin				0.22
Istaroxime	*E,Z*		oxo	0.11
2	*E*		oxo	0.056
3	*Z*		oxo	0.63
4	*E,Z*		oxo	0.37
5	*E,Z*		oxo	0.38
6	*E,Z*		oxo	28.5
7	*E,Z*		oxo	0.021
8	*E,Z*		oxo	12.0
9	*E,Z*		oxo	0.69
10	*E,Z*		oxo	0.23
11	*E,Z*		oxo	1.8
12	*E,Z*		oxo	0.10
13	*E,Z*		oxo	0.24
14	*E,Z*		oxo	0.026
15	*E*		oxo	0.016
16	*Z*		oxo	0.25
17	*E,Z*		oxo	40.5
18	*E*		oxo	1.3
19	*E,Z*		oxo	11.0
20	*E,Z*		oxo	23.0
21	*E*		oxo	33.0
22	*E*		oxo	1.1
23	*E,Z*		α-OH	0.92
24	*E,Z*		α-OH	0.91
25	*E,Z*		α-OH	0.22
26	*E,Z*		β-OH	0.85

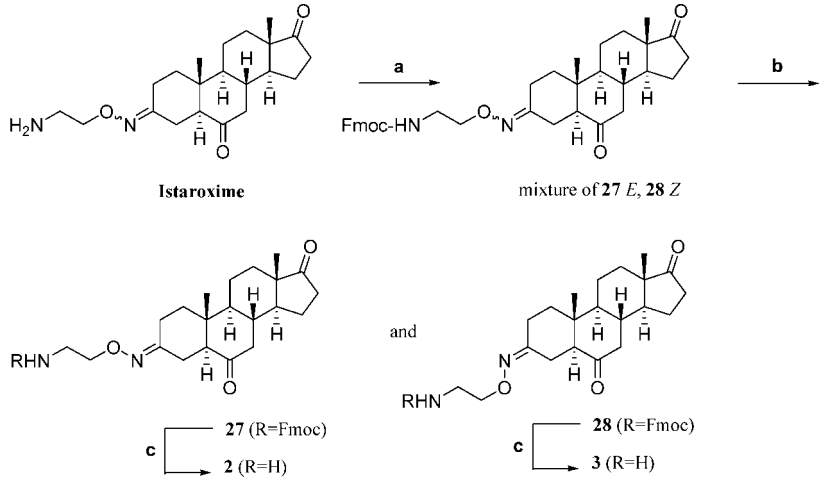
[a] Concentrations able to inhibit 50% of Na^+,K^+-ATPase enzyme activity; mean of two or three experiments.

Istaroxime, the length of the Istaroxime chain can be defined as the optimal space available for the basic chain.

Secondary Amines on Straight Chains. While the dimethylamine derivative of Istaroxime was already described and found to be almost devoid of inhibitory activity on the Na^+,K^+-ATPase,[6] we here report some secondary amines on straight chains. The monomethylamine derivative of Istaroxime, **9**, had a reduced potency of about six times; however, its homologue, **10**, had a higher activity, if compared with **9**, but still lower than Istaroxime's. A bulkier and more basic group, such as guanidinyl in compound **8**, caused an impressive drop in activity of about 100 times.

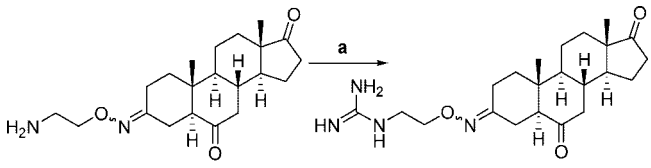
Cyclic Amines. The reduction in the degree of freedom of a molecule is a well-known method in medicinal chemistry, often used to achieve more potent compounds. As a first instance, the aminoalkyl chain of Istaroxime was "closed" to give a 3-pyrrolidinyl derivative. Among the numerous possibilities, this cyclic amine originates from the conformational analysis of the *E* isomer of Istaroxime (**2**):[11] nine conformers were obtained within 12.55 kJ mol^{-1} (3 kcal mol^{-1}), a range of energy compatible with the binding to a receptor (Figure 3a). As one of the conformers is extended, eight of nine are obviously folded, and specific/respective conformers superimposed almost perfectly on the specific conformer of the pyrrolidinyl oxime derivative (for both the *R* and *S* diastereoisomers, in the *E* form, there were five conformers); we considered the best superimposition between the conformers of **2** and those of the two pyrrolidinyl oxime derivatives on the basis of the rms. There

Scheme 2[a]



[a] Reagents and conditions. **a**: FmocCl, TEA, CH_2Cl_2, room temperature. **b**: flash chromatography. **c**: TBAF, THF, room temperature.

Scheme 3[a]



[a] Reagents and conditions. **a**: 3,5-dimethyl-1-pyrazolylformamidinium nitrate, DIPEA, EtOH, reflux.

Table 2. Inotropic and Toxic Effects in Anesthetized Guinea Pig

compound	E_{max}[a] % increase in dP/dt_{max}	ED_{max}[b] μmol/kg	ED_{80}[c] μmol/kg	deads/ treated	lethal dose/ED_{80}
digoxin	127	0.97	0.41	10/10	3.2
Istaroxime	182	5.74	1.82	7/8	25.6
2	260	6.79	1.03	8/8	20.8
3	235	12.1	3.36	4/5	22.5
14	223	8.09	2.01	1/3	12.0
15	186	2.65	0.75	3/3	8.0
16	114	43.5	27.7	0/3	>3.6
23	77	8.95		2/2	<17.0
25	216	9.62	1.64	4/5	21.0
26	256	44.8	3.92	2/2	49.1

[a] Maximal increase in dP/dt_{max}. [b] Dose inducing maximum positive inotropic effect. [c] Inotropic potency: dose increasing $+dP/dt_{max}$ by 80%, calculated from dose−response curves.

was no way to choose a priori which of the two diastereoisomeric pyrrolidinyl derivatives would have higher inhibitory potency, as differently folded conformers of Istaroxime superimposed equally well on *R*- or *S*-pyrrolidinyl derivatives (Figure 3b with the *E R*-pyrrolidinyl derivative **15**; Figure 3c with the *E* isomer of the *S*-pyrrolidinyl derivative **13**). The two different diastereoisomers were synthesized as *E*,*Z* mixtures, and the *R* isomer, **14**, proved nine times more potent than the *S* isomer, **13**. The pure *E* isomer of **14**, i.e., **15**, was 15 times more potent than the *Z* isomer, **16**, further supporting our model. The *E* isomer, **15**, was found to be three times more potent than the *E* isomer of Istaroxime, (**2**) and the *Z* isomer, **16**, was twice as potent as the *Z* isomer of Istaroxime (**3**). As shown above, secondary amines were expected to give reduced potency in comparison with primary amines; in this case, the reduction in potency caused by this modification was counteracted by the "closure" of the chain to give a cyclic, secondary amine.

In previous papers, we demonstrated that tertiary amines reduced activity by about 2 orders of magnitude, in comparison to the parent primary amines;[6,12,13] the *N*-methylpyrrolidinyl analogue **18** was about 80 times less potent than the N-unsubstituted parent compound, **15**, in agreement with what was already reported. Also, the N-methylation of the *S*-pyrrolidinyl derivative, **13**, giving compound **17**, caused a potency reduction of about 170 times. These cyclic tertiary amines had higher potencies than the dimethylamine derivative of Istaroxime (>100 μM); again, the cyclic nature of **17** and **18** had a beneficial effect on interaction with the receptor.

The 2-pyrrolidinylmethyl substituent maintains the same distance between the amino group and the oxygen of the oximic group: in both the *S* and *R* derivatives potencies were considerably reduced when compared to the 3-pyrrolidinyl analogues (**19** vs **13** and **20** vs **14**) and Istaroxime.

Restriction of the cycle to four terms, maintaining the NH at the same distance relative to the oxygen of the oximic group, gave compound **12**, with potency similar to that of Istaroxime. The enlargement of the cycle to the 3-piperidinyl derivative **21** (diastereoisomeric mixture), again maintaining the NH at the same distance relative to the oxygen of the oximic group, reduced potency; activity was also reduced with 4-piperidinyl **22**.

6-Hydroxy Derivatives. Consistent with our prediction,[6] the reduction of the keto group in position 6 led to diminished potency on the Na^+,K^+-ATPase, with the β configuration in this position more detrimental than the α stereochemistry; this fact is confirmed in the present paper by the comparisons between **23** and **10**, **24** and **13**, **25** and **14,** and between **26** and both **25** and **14**.

In Vivo Activity in Guinea Pigs. Some compounds showing high inhibitory potency on the Na^+,K^+-ATPase were investigated in vivo in the anesthetized guinea pig (Table 2). In vitro and in vivo potencies (ED_{80}) were correlated ($r^2 = 0.72$, $n = 8$, with the exclusion of compound **16**, which is an outlier, and compound **23**, which did not reach ED_{80}), thus confirming the predictivity of the in vitro test. Within the homogeneous series of the androstane derivatives, which excludes digoxin, the correlation is higher ($r^2 = 0.87$, $n = 7$).

Even though none of the tested compounds showed inotropic potencies (ED_{80}) comparable to digoxin, all displayed, most importantly, safety ratios higher than digoxin, which could be an intrinsic property of this class of cardiac androstane deriva-

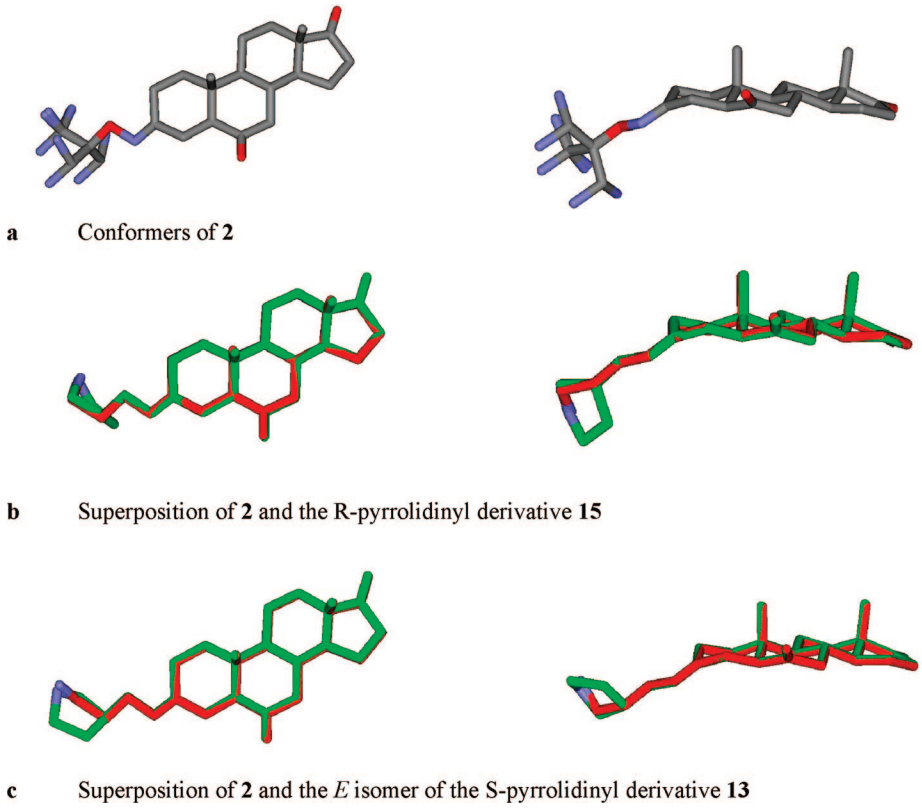


Figure 3. (a) A 3D model of conformers of compound **2**. (b) Superposition of compounds **2** (red) and **15** (green); the basic nitrogen atom of the chains are colored in cyan. (c) Superposition of compound **2** (red) and the *E* isomer of compound **13** (green); the basic nitrogen atom of the chains are colored in cyan.

tives. The reported ability of low digoxin concentrations to stimulate Ca^{2+} release from the sarcoplasmic reticulum[14] with high efficacy may partly account for its proarrhythmic properties. On the other hand, we showed that Istaroxime increases SERCA2a (Sarco/Endoplasmic Reticulum Calcium ATPase Isoform 2a) affinity for Ca^{2+}, enhancing Ca^{2+} reuptake.[15] The contribution of these mechanisms to in vivo behavior (inotropic potency, safety ratio) is not known. Investigation of SERCA2a interaction of Istaroxime's analogues was beyond the scope of the present work.

Conclusions

The compounds reported in this paper support interest in the androstane skeleton, with a proper aminoalkyloxime in position 3 as a pharmacophore for positive inotropic activity, with the likely advantage of higher safety, as compared to classic digitalis compounds. The importance of the amino group at a proper distance in the oxime chain in position 3 is demonstrated and the higher potency of the *E*, as opposed to the *Z*, isomers is confirmed; further, some of the oximic chain with a cyclic amine, here introduced for the first time, have been found to confer a higher potency than the oximic chain with a linear amine. All these confirmations or achievements encourage additional exploitation of the androstane skeleton in the search for new, positive inotropic agents, possibly safer than digoxin.

Experimental Section

General Details. General experimental details regarding chemistry and pharmacology are reported elsewhere.[12]

Chemistry. General Procedure for the Reaction of Scheme 1. To a stirred solution of the appropriate ketone (1 equiv) in THF (0.15 M), a solution of the appropriate hydroxylamines dihydrochlorides (1 equiv) in H_2O (0.30 M) was rapidly added dropwise. After 1.5 h, NaCl (7.5 equiv) was added and the mixture stirred for 10 min. The phases were separated and the aqueous phase extracted with THF (2×). The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated.

(*E*)-3-(2-Aminoethoxyimino)androstane-6,17-dione fumarate (2). To a stirred solution of **27** (2.00 g, 3.43 mmol) in dry THF (9.6 mL) at 0 °C, 1 M tetrabutylammonium fluoride in THF (4.1 mL, 4.1 mmol) was added and the mixture stirred at room temperature for 3 h. The mixture was loaded onto a flash chromatography column (SiO_2, CH_2Cl_2/MeOH/26% NH_4OH 90/10/1). The fractions containing **2** as a base were combined and concentrated under vacuum at 30 °C. MeOH was added and evaporated again to eliminate ammonia. Fumaric acid (0.40 g, 3.44 mmol) in MeOH (10 mL) was added. After addition of EtOAc (20 mL) and Et_2O (150 mL), the precipitate was filtered to give the title compound **2** (1.30 g, 80%). ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.1 (bb, 4H, NH_3^+, COOH), 6.45 (s, 2H, CH=CH), (m, 2H, CH_2−O), 3.08 (m, 1H, H-2eq), 2.98 (m, 2H, CH_2−N), 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{21}H_{32}N_2O_3 \cdot C_4H_4O_4$) C, H, N.

(*Z*)-3-(2-Aminoethoxyimino)androstane-6,17-dione fumarate (3). The title compound **3** was prepared in 83% yield from **28**, following the procedure described above for the preparation of **2**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 7.95 (bb, 4H, NH_3^+, COOH), 6.45 (s, 2H, CH=CH), 4.04 (m, 2H, CH_2−O), 3.02 (m, 1H, H-4eq), 2.96 (m, 2H CH_2−N), 0.79 (s, 3H, CH_3), 0.77(s, 3H, CH_3); white solid. Anal. ($C_{21}H_{32}N_2O_3 \cdot C_4H_4O_4$) C, H, N.

(*E*,*Z*)-3-[(*S*)-2-Amino-1-propoxyimino]androstane-6,17-dione hydrochloride (4). Prepared in 81% yield from **1a** and (*S*)-2-amino-1-propoxyamine dihydrochloride (Supporting Information, **I-a**). The title compound **4** was obtained from the crude after washing the residue with EtOAc and filtering. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.00 (bb, 3H, NH_3^+), 3.97 (m, 2H, CH_2−O), 3.40 (m, 1H, CH–N), 3.11 (m, 0.5H, H-2eq *E* isomer), 3.05 (m, 0.5H, H-4eq *Z* isomer), 1.16 (d, 3H, CH_3), 0.79 (s, 3H, CH_3), 0.78

(s, 1.5H, CH_3), 0.77 (s, 1.5H, CH_3); white solid. Anal. ($C_{22}H_{34}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-[(*R*)-2-Amino-1-propoxyimino]androstane-6,17-dione fumarate (5). Prepared in 46% yield from **1a** and (*R*)-2-amino-1-propoxyamine dihydrochloride (Supporting Information, **I-b**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions fumaric acid in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **5**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.1 (bb, 4H, NH_3^+, COOH), 6.45(s, 2H, CH═CH), 3.95 (m, 2H, CH_2−O), 3.38 (m, 1H, CH–N), 3.11 (m, 0.5H, H-2eq *E* isomer), 3.03 (m, 0.5H, H-4eq *Z* isomer), 1.14 (d, 3H, CH_3), 0.78 (s, 3H, CH_3), 0.77 (s, 3H, CH_3); white solid. Anal. ($C_{22}H_{34}N_2O_3 \cdot C_4H_4O_4$) C, H, N.

(*E*,*Z*)-3-(2-Amino-2-methyl-1-propoxyimino)androstane-6,17-dione hydrochloride (6). Prepared in 48% yield from **1a** and 2-amino-2-methyl-1-propoxyamine dihydrochloride (Supporting Information, **I-c**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **6**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 7.84 (bb, 3H, NH_3^+), 3.91 (s, 2H, CH_2−O), 3.16 (m, 0.5H, H-2eq *E* isomer), 3.08 (m, 0.5H, H-4eq *Z* isomer), 1.21 (s, 6H, C–$(CH_3)_2$), 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{23}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-(3-Amino-2-methyl-2-propoxyimino)androstane-6,17-dione fumarate (7). Prepared in 48% yield from **1a** and 1-amino-2-methyl-2-propoxyamine dihydrochloride (Supporting Information, **I-d**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, fumaric acid in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **7**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.1 (bb, 4H, NH_3^+, COOH), 6.41 (s, 2H, CH═CH), 3.07 (m, 0.5H, H-2eq *E* isomer), 3.02 (m, 0.5H, H-4eq *Z* isomer), 2.94 (m, 2H, CH_2−N), 1.22 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 0.79 (s, 3H, CH_3), 0.78 (s, 1.5H, CH_3), 0.77 (s, 1.5H, CH_3); white solid. Anal. ($C_{23}H_{36}N_2O_3 \cdot C_4H_4O_4$) C, H, N.

(*E*,*Z*)-3-(2-Guanidinoethoxyimino)androstane-6,17-dione nitrate (8). Istaroxime hydrochloride[6] (0.50 g, 1.26 mmol) and 3,5-dimethyl-1-pyrazolylformamidinium nitrate (0.30 g, 1.49 mmol) were dissolved in EtOH (7.5 mL), then DIPEA (0.43 mL, 2.47 mmol) was added and the mixture refluxed for 1.5 h. After cooling, the resulting slurry was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 90:10:1): the solid obtained was washed with water and then dried at 40 °C under vacuum to give the title compound **8** (0.27 g, 46%). ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 7.38 (t, 1H, NH), 7.02 (m, 4H, C−$N_2H_4^+$), 4.00 (m, 2H, CH_2O), 3.35 (m, 2H, CH_2N), 3.02 (m, 0.5H, H-2eq *E* isomer), 2.97 (m, 0.5H, H-4eq *Z* isomer), 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{22}H_{34}N_4O_3 \cdot HNO_3$) C, H, N.

(*E*,*Z*)-3-(2-*N*-Methylaminoethoxyimino)androstane-6,17-dione hydrochloride (9). Prepared in 64% yield from **1a** and 2-*N*-methylaminoethoxyamine dihydrochloride (Supporting Information, **I-e**). The title compound **9** was obtained from the crude, after washing the residue with EtOAc and crystallizing from 96% EtOH. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.66 (bb, 2H, NH_2^+), 4.14 (m, 2H, CH_2−O), 3.14 (m, 2H, CH_2−N), 3.08 (m, 0.5H, H-2 *E* isomer), 3.00 (m, 0.5H, H-4 *Z* isomer), 2.55 (s, 1.5H, NCH_3), 2.55 (s, 1.5H, NCH_3), 0.79 (s, 3H, CH_3), 0.78 (s, 1.5H, CH_3), 0.77 (s, 1.5H, CH_3); white solid. Anal. ($C_{22}H_{34}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-(3-*N*-Methylaminopropoxyimino)androstane-6,17-dione hydrochloride (10). Prepared in 63% yield from **1a** and 3-*N*-methylaminopropoxyamine dihydrochloride (Supporting Information, **I-f**). The title compound **10** was obtained from the crude after washing the residue with EtOAc and filtering. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.56 (bb, 2H, NH_2^+), 3.97 (m, 2H, CH_2−O), 3.00 (m, 0.5H, H-2eq *E* isomer), 2.93 (m, 0.5H, H-4eq *Z* isomer), 2.88 (m, 2H, CH_2−N), 2.51 (s, 3H, CH_3−N), 0.79 (s, 3H, CH_3), 0.78 (s, 1.5H, CH_3), 0.77(s, 1.5H, CH_3); white solid. Anal. ($C_{23}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-(4-Aminobutoxyimino)androstane-6,17-dione hydrochloride (11). Prepared in 64% yield from **1a** and 4-aminobutoxyamine dihydrochloride.[12] The title compound **11** was obtained from the crude after washing the residue with EtOAc and filtering. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 7.74 (bb, 3H, NH_3^+), 3.92 (m, 2H, CH_2−O), 2.99 (m, 0.5H, H-2eq *E* isomer), 2.94 (m, 0.5H, H-4eq *Z* isomer), 2.72 (m, 2H, CH_2−N), 0.79 (s, 3H, CH_3), 0.78 (s, 1.5H, CH_3), 0.77 (s, 1.5H, CH_3); white solid. Anal. ($C_{23}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-(3-Azetidinyloxyimino)androstane-6,17-dione fumarate (12). Prepared in 80% yield from **1a** and 3-azetidinyloxyamine dihydrochloride (Supporting Information, **I-g**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, fumaric acid in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **12**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.00 (bb, 4H, NH_3^+, COOH), 6.45 (s, 2H, CH═CH), 4.88 (m, 1H, CH−O), 4.13 (m, 2H, CH_2−N), 3.87 (m,2H, CH_2−N), 3.05 (m, 0.5H, H-2eq *E* isomer), 2.99 (m, 0.5H, H-4eq *Z* isomer), 0.79 (s, 3H, CH_3), 0.79 (s, 3H, CH_3); white solid. Anal. ($C_{22}H_{32}N_2O_3 \cdot C_4H_4O_4$) C, H, N.

(*E*,*Z*)-3-[3-(*S*)-Pyrrolidinyloxyimino]androstane-6,17-dione hydrochloride (13). Prepared in 78% yield from **1a** and 3-(*S*)-pyrrolidinyloxyamine dihydrochloride (Supporting Information, **I-h**). The title compound **13** was obtained from the crude, after washing the residue with EtOAc, Et_2O, and filtering. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 9.23 (bb, 2H, NH_2^+), 4.74 (m, 1H, CH–O), 3.15 (m, 4H, CH_2−N–CH_2), 3.01 (m, 0.5H, H-2eq *E* isomer), 2.96 (m, 0.5H, H-4eq *Z* isomer), 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{23}H_{34}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-[3-(*R*)-Pyrrolidinyloxyimino]androstane-6,17-dione hydrochloride (14). Prepared in 72% yield from **1a** and 3-(*R*)-pyrrolidinyloxyamine dihydrochloride (Supporting Information, **I-i**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **14**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 9.20 (bb, 2H, NH_2^+), 4.75 (m, 1H, CH–O), 3.18 (m, 4H, CH_2−N–CH_2), 3.03 (m, 0.5H, H-2eq *E* isomer), 2.96 (m, 0.5H, H-4eq *Z* isomer), 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{23}H_{34}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*)-3-[3-(*R*)-Pyrrolidinyl]oxyiminoandrostane-6,17-dione hydrochloride (15). (*E,Z*) 3-[3-(*R*)-pyrrolidinyloxyimino]androstane-6,17-dione hydrochloride (**14**, 0.65 g) was suspended in EtOAc (150 mL) and stirred for 3 h. After filtration, the procedure was repeated on the solid to give the title compound **15** (0.30 g, 46%). ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 9.26 (bb, 2H, NH_2^+), 4.74 (m, 1H, CH–O), 3.18 (m, 4H, CH_2−N–CH_2) 3.03 (m, 1H, H-2eq), 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{23}H_{34}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*Z*)-3-[3-(*R*)-Pyrrolidinyl]oxyiminoandrostane-6,17-dione hydrochloride (16). The mother liquor of the first filtration reported for the preparation of compound **15** was evaporated to dryness. The residue was dissolved in EtOH, filtered on charcoal, and the filtrate concentrated to small volume; the solid was collected by filtration to give the title compound **16** (0.25 g, 38%). ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 9.22 (bb, 2H, NH_2^+), 4.75 (m, 1H, CH–O), 3.18 (m, 4H, CH_2−N–CH_2), 2.96 (m, 1H, H-4eq), 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{23}H_{34}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-[3-(*S*)-(1-Methyl)pyrrolidinyloxyimino]androstane-6,17-dione hydrochloride (17). Prepared in 45% yield from **1a** and 3-(*S*)-(1-methyl)pyrrolidinyloxyamine dihydrochloride (Supporting Information, **I-j**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **17**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 10.58 (bb, 1H, NH^+), 4.76 (m, 1H, CH–O), 3.30 (m, 4H, CH_2−N–CH_2), 3.05 (m, 0.5H,

H-2eq *E* isomer), 2.97 (m, 0.5H, H-4eq *Z* isomer), 2.75 (s, 3H, CH_3−N), 0.79 (s, 3H, CH_3), 0.78 (s, 1.5H, CH_3), 0.77 (s, 1.5H, CH_3); white solid. Anal. ($C_{24}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*)-3-[3-(*R*)-(1-Methyl)pyrrolidinyloxyimino]androstane-6,17-dione (18). Prepared in 85% yield from **1a** and 3-(*R*)-(1-methyl)pyrrolidinyloxyamine dihydrochloride (Supporting Information, **I-k**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). The solid residue from the concentrated fractions was suspended in EtOAc and then collected by filtration to give the title compound **18**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 4.57 (m, 1H, CH–O), 3.00 (m, 1H, H-2eq), 2.19 (s, 3H, CH_3−N), 0.78 (s, 3H, CH_3), 0.77 (s, 3H, CH_3); light yellow solid. Anal. ($C_{24}H_{36}N_2O_3$) C, H, N.

(*E*,*Z*)-3-[2-(*S*)-Pyrrolidinylmethoxyimino]androstane-6,17-dione hydrochloride (19). Prepared in 57% yield from **1a** and 2-[(*S*)-pyrrolidinyl]methoxyamine dihydrochloride (Supporting Information, **I-l**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **19**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 9.55 (bb, 1H, NH^+), 8.79 (bb, 1H, NH^+), 4.12 (m, 2H, CH_2−O), 3.69 (m, 1H, CH–N), 3.12 (m, 2H, CH_2−N), 3.10 (m, 0.5H, H-2 *E* isomer), 3.01 (m, 0.5H, H-4 *Z* isomer), 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{24}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-[2-(*R*)-Pyrrolidinylmethoxyimino]androstane-6,17-dione hydrochloride (20). Prepared in 57% yield from **1a** and 2-[(*R*)-pyrrolidinyl]methoxyamine dihydrochloride (Supporting Information, **I-m**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **20**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 9.40 (bb, 1H, NH^+), 8.84 (bb, 1H, NH^+), 4.11 (m, 2H, CH_2−O), 3.71 (m, 1H, CH–N), 3.10 (m, 2H, CH_2−N), 3.08 (m, 0.5H, H-2 *E* isomer), 3.02 (m, 0.5H, H-4 *Z* isomer), 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{24}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*)-3-[3-(*R*,*S*)-Piperidinyloxyimino]androstane-6,17-dione hydrochloride (21). Prepared in 76% yield from **1a** and 3-(*RS*)-piperidinyloxyamine dihydrochloride (Supporting Information, **I-n**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **21**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.68 (bb, 2H, NH_2^+), 4.21 (m, 1H, CH–O), 3.10 (m, 5H, CH_2−N–CH_2 + H-2eq), 0.79 (s, 3H, CH_3), 0.79 (s, 3H, CH_3); white solid. Anal. ($C_{24}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*)-3-(4-Piperidyloxyimino)androstane-6,17-dione hydrochloride (22). Prepared in 60% yield from **1a** and 4-piperidyloxyamine dihydrochloride (Supporting Information, **I-o**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **22**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.68 (bb, 2H, NH_2^+), 4.17 (m, 1H, CH–O), 3.05 (m, 5H, CH_2−N–CH_2 and H-2eq) 0.79 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{24}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-(3-*N*-Methylaminopropoxyimino)-6α-hydroxyandrostane-17-one hydrochloride (23). Prepared in 57% yield from **1b** and 3-*N*-methylaminopropoxyamine dihydrochloride (Supporting Information, **I-f**). The title compound **23** was obtained from the crude after washing the residue with EtOAc, Et_2O, and filtering. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 8.75 (bb, 2H, NH_2^+), 4.55 (d, 0.5H, OH E isomer), 4.51 (d, 0.5H, OH Z isomer), 3.96 (m, 2H, CH_2−O), 3.41 (m, 0.5H, H-4eq *Z* isomer), 3.29 (m, 1H, CH–O), 2.97 (m, 0.5H, H-2eq *E* isomer), 2.89 (m, 2H, CH_2−N), 2.51 (s, 3H, CH_3−N), 0.86 (s, 1.5H, CH_3), 0.85 (s, 1.5H, CH_3), 0.77 (s, 1.5H, CH_3); white solid. Anal. ($C_{23}H_{38}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-[3-(*S*)-Pyrrolidinyl]oxyimino-6α-hydroxyandrostane-17-one hydrochloride (24). Prepared in 70% yield from **1b** and 3-(*S*)-pyrrolidinyloxyamine dihydrochloride (Supporting Information, **I-h**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **24**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 9.12 (bb, 2H, NH_2^+), 4.72 (m, 1H, CH–O), 4.54 (d, 1H, OH), 3.40 (m, 0.5H, H-4eq *Z* isomer), 3.25 (m, 5H, CH_2−N–CH_2 and CH–O), 2.99 (m, 0.5H, H-2eq *E* isomer), 0.86 (s, 1.5H, CH_3), 0.85 (s, 1.5H, CH_3), 0.77 (s, 3H, CH_3); white solid. Anal. ($C_{23}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-[3-(*R*)-Pyrrolidinyl]oxyimino-6α-hydroxyandrostane-17-one hydrochloride (25). Prepared in 70% yield from **1b** and 3-(*R*)-pyrrolidinyloxyamine dihydrochloride (Supporting Information, **I-i**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **25**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 9.15 (bb, 2H, NH_2^+), 4.73 (m, 1H, CH–O), 4.52 (d, 1H, OH), 3.40 (m, 0.5H, H-4eq *Z* isomer), 3.25 (m, 5H, CH_2−N–CH_2 and CH–O) 2.99 (m, 0.5H, H-2eq *E* isomer), 0.87 (s, 1.5H, CH_3), 0.85 (s, 1.5H, CH_3), 0.77 (s, 3H, CH_3); white solid. Anal. ($C_{23}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

(*E*,*Z*)-3-[3-(*R*)-Pyrrolidinyl]oxyimino-6β-hydroxyandrostane-17-one hydrochloride (26). Prepared in 80% yield from **1c** and 3-(*R*)-pyrrolidinyloxyamine dihydrochloride (Supporting Information, **I-i**). The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3 9:1:0.1). To the concentrated fractions, 5 M HCl in EtOAc was added. After dilution with Et_2O, the solid was collected by filtration to give the title compound **26**. ^{1}H NMR (300 MHz, DMSO-d_6, ppm from TMS): δ 9.25 (bb, 2H, NH_2^+), 4.73 (m, 1H, CH–O), 4.45 (d, 0.5H, OH), 4.43 (d, 0,5H, OH), 3.65 (m, 1H, CH–O), 3.17 (m, 4H, CH_2−N–CH_2), 3.00 (m, 0.5H, H-2eq *E* isomer), 2.76 (m, 0.5H, H-4eq *Z* isomer), 0.96 (s, 3H, CH_3), 0.78 (s, 3H, CH_3); white solid. Anal. ($C_{23}H_{36}N_2O_3 \cdot HCl$) C, H, N, Cl.

[(*E*)-2-(6,17-Dioxoandrostane-3-ylideneaminooxy)ethyl]carbamic Acid 9*H*-Fluoren-9-ylmethyl ester (27) and [(*Z*)-2-(6,17-Dioxoandrostane-3-ylideneaminooxy)ethyl]carbamic Acid 9*H*-fluoren-9-ylmethyl ester (28). A solution of 9-fluorenylmethoxycarbonyl chloride (10.18 g, 39.3 mmol) in CH_2Cl_2 (40 mL) was added dropwise to a stirred solution of Istaroxime hydrochloride[6] (13.00 g, 32.7 mmol) in CH_2Cl_2 (90 mL) and Et_3N (10.0 mL, 71.7 mmol) in an ice bath. After completing the addition, the mixture was stirred in an ice bath for 10 min. Water (200 mL) was carefully added dropwise and the mixture extracted with CH_2Cl_2 (2 × 100 mL). The organic phase was washed with 0.1N HCl, 5% aqueous $NaHCO_3$, H_2O, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by flash chromatography and divided into four columns (SiO_2; CH_2Cl_2/acetone 95/5) to give, as overall result, compounds **27** (7.60 g, 40%) and **28** (7.60 g, 40%). **27**: ^{1}H NMR (300 MHz, acetone-d_6, ppm from TMS) δ 7.90−7.25 (m, 8H, aromatics), 6.53 (bt, 1H, NH), 4.28 (m, 3H, CH−CH_2−O), 4.05 (m, 2H, CH_2−O), 3.41 (m, 2H, CH_2−N), 3.16 (m, 0.5H, H-2eq), 0.85 (s, 3H, CH_3), 0.84 (s, 3H, CH_3). **28**: ^{1}H NMR (300 MHz, acetone-d_6, ppm from TMS) δ 7.90−7.25 (m, 8H, aromatics), 6.56 (bt, 1H, NH), 4.25 (m, 3H, CH−CH_2−O), 4.05 (m, 2H, CH_2−O), 3.41 (m, 2H, CH_2−N), 3.11 (m, 0.5H, H-4eq), 0.85 (s, 3H, CH_3), 0.84 (s, 3H, CH_3).

Conformational Energy Calculations. The conformational analyses of **2**, **15**, and the *E* isomer of **13** were performed using the OPLS2005 all-atom force field as implemented in the MacroModel 9.1 program; PR conjugate gradient was used in all the minimization steps, with the derivative convergence set to 0.05 (kJ/mol)/Å, with a maximum of 5000 iterations. The Monte Carlo multiple minimum method was used in the conformational search (OPLS2005, 5000 steps, torsion rotations allowed on the amino chain). All conformations within 50 kJ/mol of the identified lowest energy conformer were minimized again (Multiple Minimization routine in the program) using OPLS2005 in conjunction with the GB/SA continuum model to simulate water solvation.

Supporting Information Available: Elemental analysis results for target compounds, experimental details of the synthesis and characterization data for the required hydroxylamines. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM800257S