Uniwersytet w Białymstoku
Wydział Chemii

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Selenosteroidy – metody syntezy przy wykorzystaniu nowych i klasycznych reagentów selenoorganicznych

Rozprawa doktorska

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„Fortis fortuna adiuvat”

Terencjusz

Wykaz skrótów

Ac	acetyl
APAF	czynnik aktywujący peptydazę apoptyczną
APTS	3-aminopropylo trietoksysilan
BID	białko proapoptyczne
DCM	dichlrometan
DIBAL	wodorek diizobutyloglinu
DIPEA	diizopropyloetyloamina
DMAP	4-dimetyloaminopirydyna
DOX	doksorubicyna
DTT	ditiotreitol
Et	etyl
FT3	trijodotyronina
FT4	tyronina
GI_{50}	stężenie hamujące wzrost 50% komórek
GPX	peroksydaza glutationowa
H9c2	linia komórkowa tkanki sercowej szczura
HeLA	linia komórkowa raka szyjki macicy
IC_{50}	stężenie hamujące 50% danego czynnika
LDA	diizopropyloamidek litu
MCF-7	linia komórkowa nabłonkowego raka piersci
mCPBA	kwas meta-chloroperoksybenzoesowy
MIC	najniższe stężenie inhibitora
MsCl	chlorek mesylu
NMR	spektroskopia magnetycznego rezonansu magnetycznego
PDHB	dehydrogenaza pirogronowa
Py	pirydyna
ROS	reaktywne formy tlenu
THF	tetrahydrofuran
TsCl	chlorek tosylu

Spis treści

1. Streszczenie w języku polskim

W ostatnich latach wzrosło zainteresowanie nad syntezą związków organicznych zawierających w swojej budowie selen. Selen ze względu na swoje biologiczne właściwości, pełni ważną rolę w organizmie ssaków np. peroksydaza glutationowa zawierająca w swoim centrum aktywnym selen odpowiada za regulację stresu oksydacyjnego i ochronę przed rodnikowymi formami tlenu (ROS). Innym ważnym związkiem zawierającym w swojej budowie selen jest dejodynaza czyli enzym odpowiadający za transformację tyroksyny (fT4) w aktywną formę trijodotyroniny (fT3) czyli hormonu wytwarzanego przez tarczycę.

Selenosteroidy, które są tematem niniejszej dysertacji, są nieliczną grupą związków. W literaturze istnieje niewiele opisów ich syntezy, a jeszcze mniej dotyczących ich właściwości biologicznych.

W ramach prowadzonych przeze mnie badań, opracowałem metody otrzymywania nowych pochodnych steroidowych zawierających ugrupowania selenoorganiczne. Do przeprowadzonych syntez substrami łatwo dostępne steroidy oraz ich pochodne. Funkcjonalizację steroidów zastosowałem w oparciu o wykorzystanie reagenta Santiego (PhSeZnCl), system dwufazowy, w którym reagentem są diselenki oraz sprzęganie elektrofilowych reagentów selenoorganicznych z terminalnymi alkinami. Otrzymane związki zostały przebadane pod kątem właściwości biologicznych we współpracy z zakładem Farmakologii Doświadczalnej Uniwersytetu Medycznego w Białymstoku oraz z Wydziałem Biologii Uniwersytetu w Białymstoku. Wyniki niniejszej rozprawy zostały opisane w trzech publikacjach o zasięgu międzynarodowym oraz były prezentowane na konferencjach o zasięgu krajowym i międzynarodowym.

2. Streszczenie w języku angielskim

In recent years there has been an increased interest in the synthesis of organic compounds containing selenium or selenoorganic fragments. Selenium, due to its biogenic properties, plays an important role in mammalian organisms. Glutathione peroxidase, which contains selenium in its active center, is responsible for regulating oxidative stress and protecting against reactive oxygen species (ROS). Another important compound containing selenium in its structure is deiodinase, an enzyme responsible for the conversion of thyroxine (fT4) into the active form of triiodothyronine (fT3), a hormone produced by the thyroid gland.

Selenosteroids, which are the subject of this dissertation, are a relatively unexplored group of compounds. There are few descriptions of their synthesis in the literature, and even fewer regarding their biological properties.

As part of my research, I have developed methods for obtaining new steroid derivatives containing selenoorganic groups. I used readily available steroids or their derivatives for the conducted syntheses. Steroid functionalization was carried out using Santi's reagent (PhSeZnCl), a two-phase system utilizing diselenides as reagents, and the coupling of electrophilic selenoorganic reagents with terminal alkynes. The obtained compounds were examined for their biological properties in collaboration with the Department of Experimental Pharmacology at the Medical University of Białystok and the Faculty of Biology at the University of Białystok. The results of this dissertation have been described in three internationally published papers and have been presented at national and international conferences.

3. Cel Pracy

Celem niniejszej pracy było opracowanie efektywnej metody funkcjonalizacji steroidów w związki selenoorganiczne. Steroidy stanowią ważną grupę związków w świecie ssaków pełniąc szereg różnych funkcji biologicznych np. cholesterol, będący ważnym elementem błony komórkowej oraz substratem w biosyntezie hormonów męskich i żeńskich. W medycynie steroidy mają zastosowanie jako leki przeciwalergiczne, przeciwzapalne i immunosupresyjne. Związki selenoorganiczne

znane są ze swoich właściwości przeciwoksydacyjnych, przeciwzapalnych np. ebselen. Połączenie tych dwóch różnych klas związków spowoduje powstanie nowych pochodnych o nieznanych właściwościach biologicznych.

W syntezie selenosteroidów postanowiłem wykorzystać reakcję otwierania epoksydów przy użyciu PhSeZnCl, reakcję Michaela oraz sprzęganie terminalnych alkinów steroidowych z elektrofilowymi reagentami selenoorganicznymi katalizowane solami miedzi(I). Otrzymane związki zostały poddane badaniom biologicznym pod kątem właściwości antybakteryjnych i przeciwnowotworowych.

Należy podkreślić, że steroidy jako grupa związków są wymagającymi cząsteczkami ze względu na specyficzną strukturę, sztywność oraz obecność reaktywnych grup funkcyjnych. Opisane w literaturze metody funkcjonalizacji związków w podstawniki zawierające selen wymagały modyfikacji gdy substratem był steroid.

Uzyskane wyniki opublikowano w 4 artykułach (P1-P4):

P1 P A. Grzes, K. Niemirowicz-Laskowska, H Car, „Selenosteroids - promising hybrid compounds with pleiotropic biological activity: synthesis and biological aspects" *Journal of Steroid Biochemistry and Molecular Biology* 213 **(2021**) Impact factor 5.011

P2 I. Jastrzebska, S. Mellea, V. Salerno, **P. A. Grześ**, L. Siergiejczyk, K. Niemirowicz-Laskowska, R. Bucki, B. Monti, C. Santi. „PhSeZnCl in the Synthesis of Steroidal β-Hydroxy-Phenylselenides Having Antibacterial Activity",

Int. J. Mol. Sci., 20(9), 21212019 (**2019**) *IF 6.208*

P3 P. A. Grześ, B. Monti, N. Wawrusiewicz-Kurylonek, L. Bagnoli, L. Sancineto, I. Jastrzebska, C. Santi, Simple Zn-Mediated Seleno- and Thio-Functionalization of Steroids at C-1 Position, . *Int. J. Mol. Sci*. **2022**, 23, 3022. *IF 6.208*

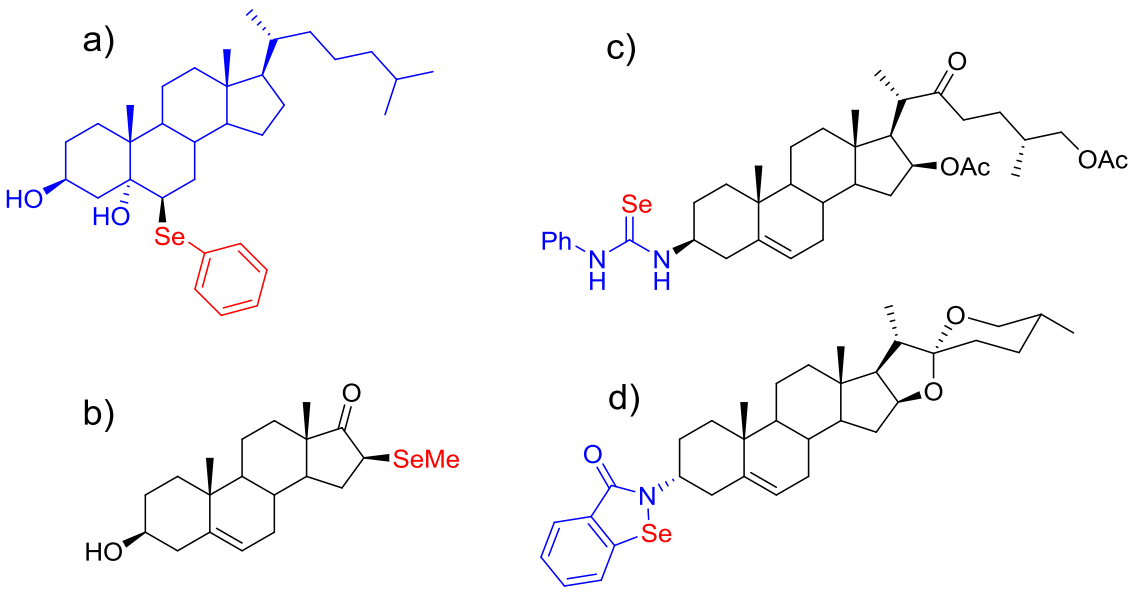
P4 P. A. Grzes, A. Sawicka, K. Niemirowicz-Laskowska, P. Wielgat, D. Sawicka, H. Car, I. Jastrzebska „Metal-promoted synthesis of steroidal ethynyl selenides having anticancer activity" JourJournal of Steroid Biochemistry and Molecular Biology 227 (**2023**) 106232 *IF 5.011*

4. Część literaturowa

W ciągu ostatnich dekad zaobserwowano wzrost zainteresowania syntezą związków zawierających w strukturze selen ze względu na obiecującą aktywność biologiczną [1-4]. Selen, jako pierwiastek ważny biologicznie, występuje u ssaków w postaci seleno-enzymów oraz białek. Przykładem takiego enzymu jest peroksydaza glutationowa zawierająca w swojej strukturze selenocysteinę. Jej zadaniem jest ochrona

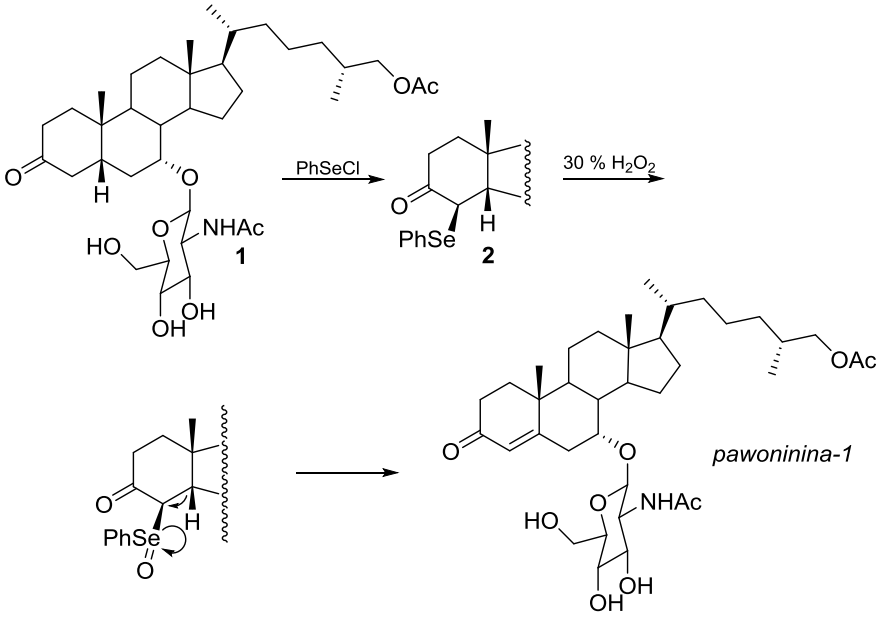
układu krwionośnego przed reaktywnymi formami tlenu [5-7]. Ze względu na liczne właściwości terapeutyczne i ochronne związków selenu, zostały zaprojektowane i zsyntezowane związki na bazie steroidów posiadające w swojej budowie ugrupowanie selenoorganiczne. Mimo że selenosteroidy nie występują w naturze, to wiele z nich wykazuje właściwości mimetyczne peroksydazy glutationowej, przeciwutleniające, przeciwnowotworowe i przeciwdrobnoustrojowe [8]. Selenosteroidy otrzymywane są poprzez przyłączenie ugrupowania zawierającego atom selenu do steroidu. Ze względu na swoją budowę związki te możemy podzielić na dwie grupy.

W pierwszej, atom selenu jest bezpośrednio związany z cząsteczką steroidu tworząc odpowiedni alkilo- lub arylo-selenek (Schemat 1. a,b). W drugiej grupie selen jest wprowadzany poprzez ugrupowanie np selenomocznikowe czy benzoseleneazolowe (Schemat1.c,d).



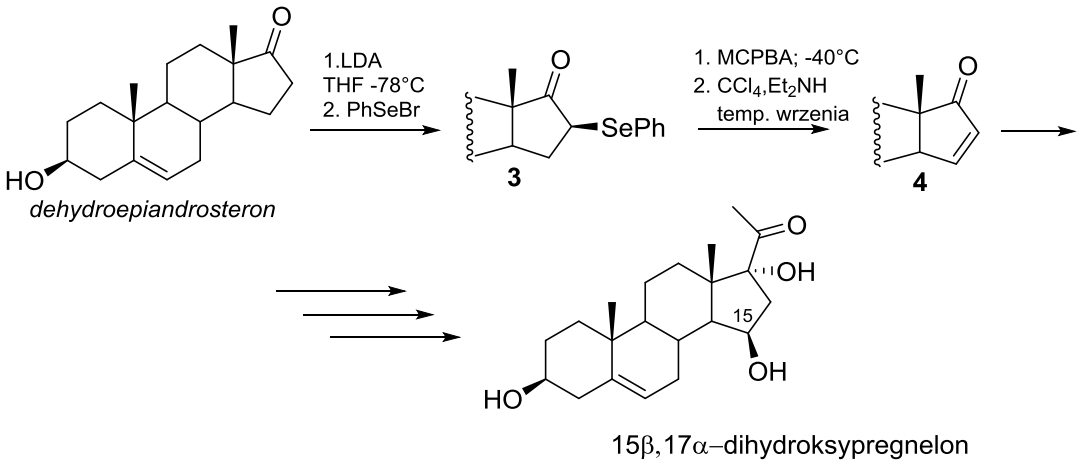
Schemat 1. Podział selenosteroidów ze względu na ich budowę.

Początkowo selenosteroidy nie były traktowane jako nowa klasa związków o określonych właściwościach biologicznych. Doskonałym przykładem jest synteza pawonininy-1, związku o właściwościach odstraszającym rekiny. Na jednym z końcowych etapów wprowadzana jest grupa fenyloselenowa w wyniku addycji elektrofilowej w pozycję α do grupy karbonylowej steroidu **1**. Otrzymana selenopochodna **2** poddawana jest reakcji z nadtlenkiem wodoru. Po czym następuje eliminacja PhSeOH z utworzeniem olefiny [9] (Schemat 2.).



Schemat 2. Końcowy etap otrzymywania pawonininy-1.

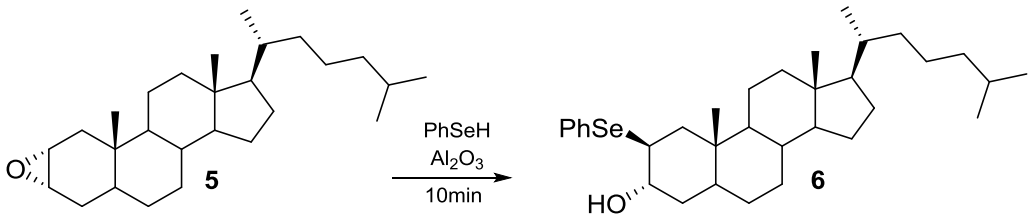
Innym przykładem zastosowania selenosteroidów jako produktu przejściowego **4** jest synteza 15β-hydroksysteroidów będących unikalnymi metabolitami powstającymi w ostatnim trymestrze ciąży ludzkiej. W pierwszym etapie działając na dehydroepiandrosteron LDA a następnie bromkiem fenyloselenylowym otrzymano pochodną **3**. Uzyskany związek poddano reakcji z MCPBA w -40°C, a następnie z dietyloaminą otrzymując olefinę **4** (Schemat 3) [10-11].



Schemat 3. Synteza 15β,17α-dihydroksypregnelonu.

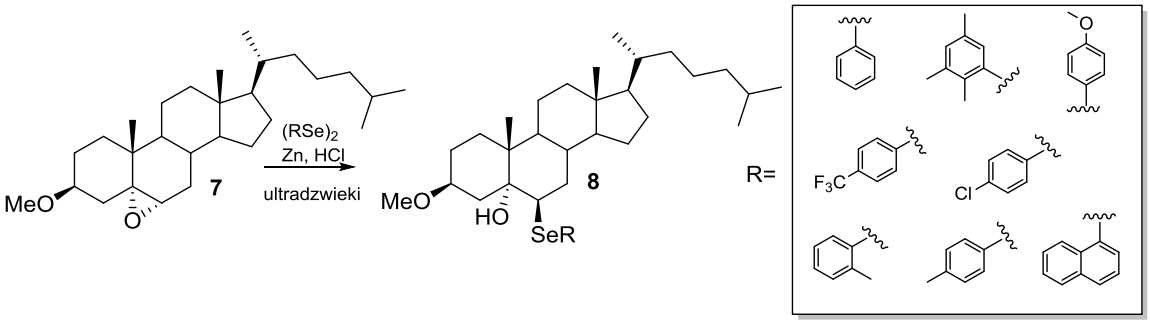
Wydajną metodą otrzymywania selenosteroidów jest reakcja otwierania pierścienia epoksydowego. Steroidowy epoksyd **5** został otwarty w obecności selenolu

i obojętnego tlenku glinu(III) typu Brockmann I. W wyniku tej reakcji uzyskano hydroksy-selenek **6** z wydajnością 72% (Schemat 4) [12].



Schemat 4. Synteza selenosteroidu **6.**

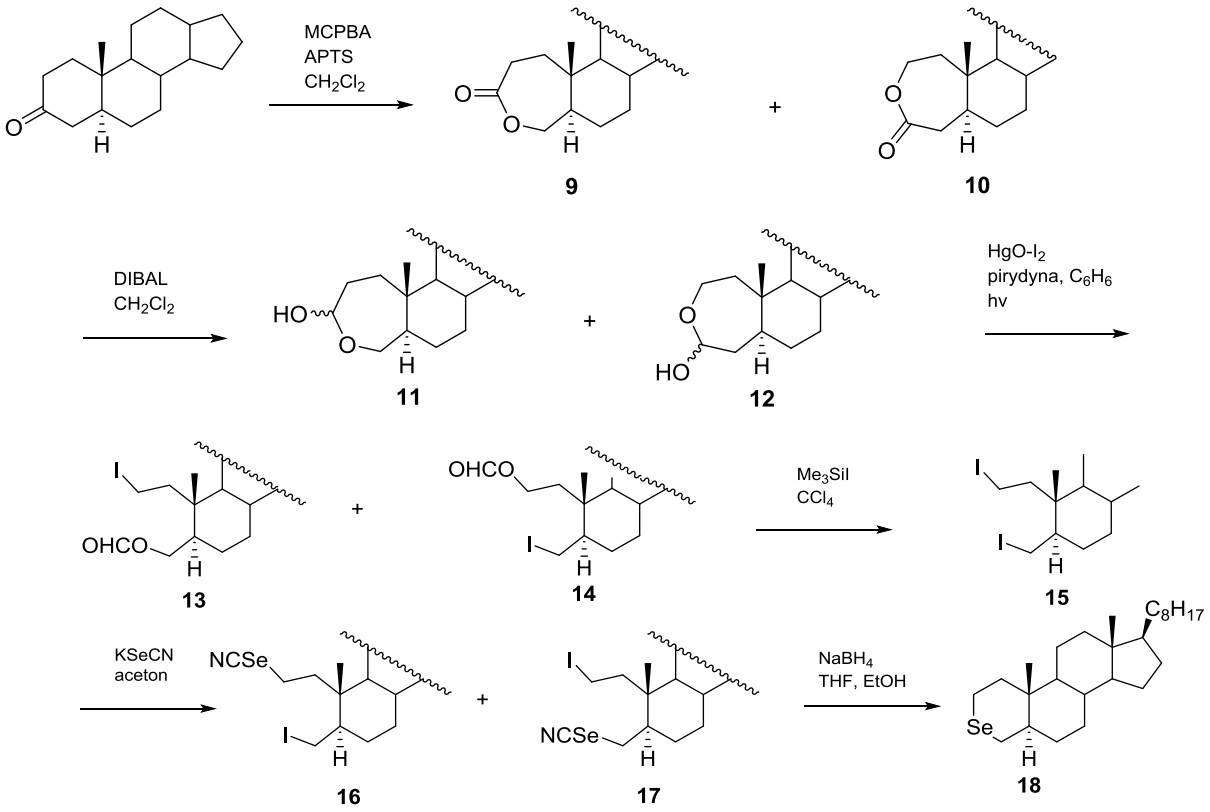
Zmodyfikowaną metodę otrzymywania selenosteroidów poprzez otwieranie oksiranów zaproponował Braga *et.al*. W pierwszej metodzie epoksyd **7** reagował z diselenkiem poddanym wcześniejszej redukcji z borowodorkiem sodu[13]. W drugiej metodzie, selenol został uzyskany poprzez działanie układu Zn/HCl wspomagany ultradźwiękami. W ten sposób uzyskano szereg transhydroksyselenków steroidowych **8** (Schemat 5) [14].



Schemat 5. Otrzymywanie selenosteroidów w reakcji otwierania pierścienia epoksydowego.

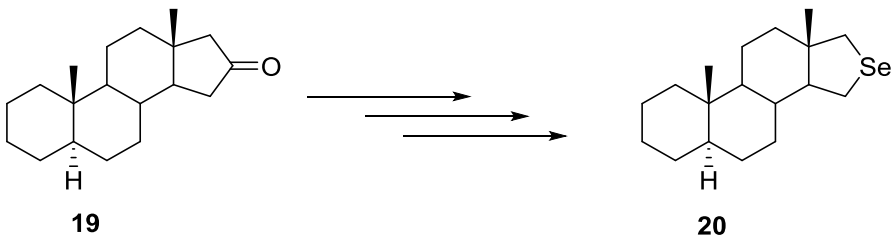
W 1990 Suginome, *et.al* otrzymał selenosteroidy zawierające atom selenu w szkielecie steroidu. W pierwszym etapie 5α-cholestan-3-on utleniono przy użyciu MCPBA uzyskując lakton **9** i **10**. Uzyskane laktony zredukowano przy pomocy DIBAL otrzymując laktole **11** i **12**, które przekształcono do jodopochodnych **13** i **14** z wykorzystaniem HgO-I_2. Następnym etapem była transformacja uzyskanych związków do dijodopochodnej **15** przy użyciu jodku trimetylosililowego. W przedostatnim etapie uzyskano selenocyjanki **16-17** poprzez dodanie jednego

ekwiwalentu KSeCN rozpuszczonego w acetonie do jodopochodnej **15**. Ostatnim etapem otrzymywania 3-selenocholestanu była redukcja związków **16** i **17** przy użyciu $NaBH_4$ (Schemat 6) [15].



Schemat 6. Synteza 3-selenocholestanu z 5α-cholesten-3-onu.

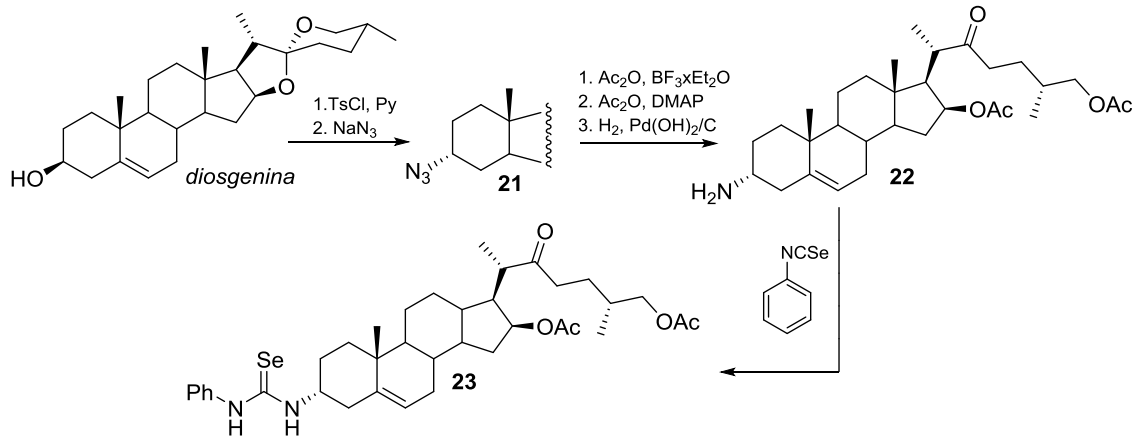
Analogiczną ścieżkę syntezy zastosowano do otrzymania selenosteroidu z wbudowanym atomem selenu w pierścieniu D **20** wykorzystując jako substrat 5α-androstan-16-on **19**. (Schemat 7).



Schemat 7. Synteza 16-selenoandrostanu.

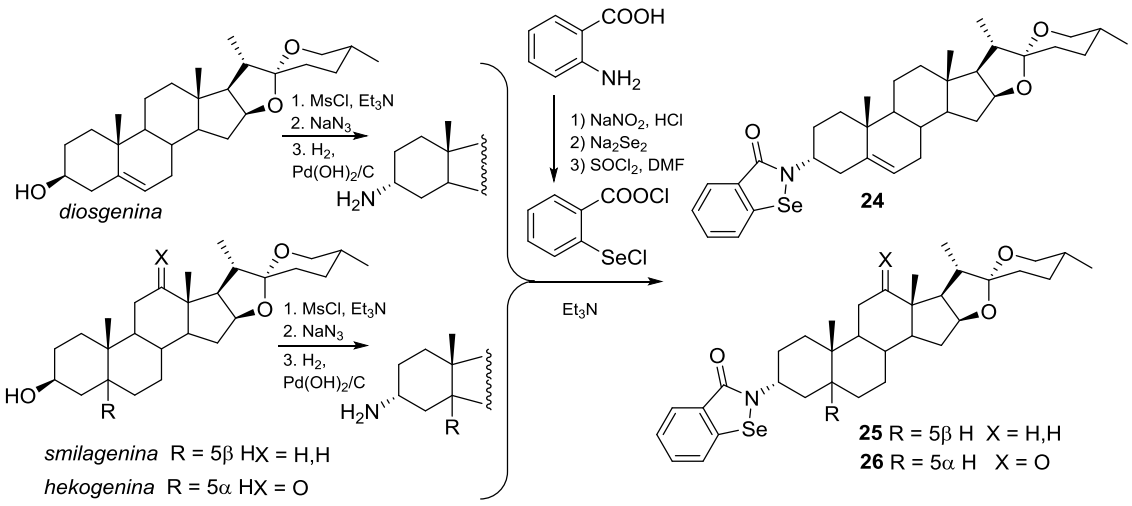
Innym z otrzymanych selenosteroidów jest selenomocznikowa pochodna **23** otrzymana z diosgeniny. W pierwszym etapie grupa hydroksylowa w pozycji *C-3* jest

poddawana tosylowaniu, a następnie substytucji z NaN_3. W wyniku tych reakcji otrzymano azydek steroidowy **21** z odwróconą konfiguracją na węglu *C-3*. W wyniku następnych przekształceń uzyskano amino-steroid **22**, który poddano reakcji z izocyjanianem fenylu uzyskując oczekiwaną pochodną **23**. (Schemat 8) [16].



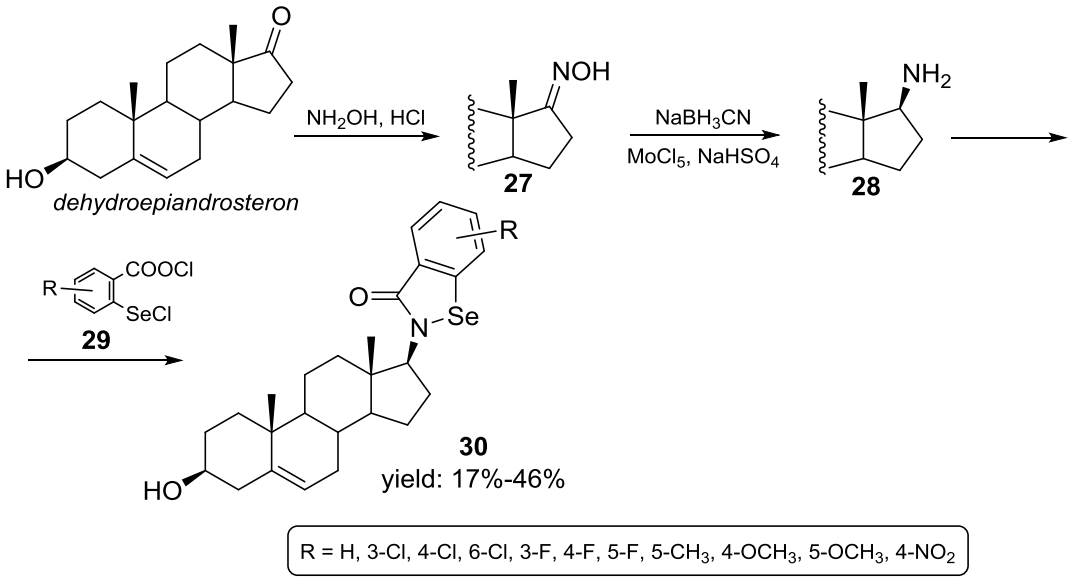
Schemat 8. Otrzymywanie seleno-mocznikowej pochodnej **23** z wykorzystaniem diosgeniny.

Innymi selenosteroidami będącymi mimetykami peroksydazy glutationowej (GPx) są steroidowe pochodne ebselenu. W pierwszym etapie dokonano transformacji grupy hydroksylowej w pozycji *C-3* na grupę aminową. Równolegle otrzymano fragment ebselenowy. W pierwszym etapie kwas 2-aminobenzoesowy poddawany jest diazowaniu z azotanem(III) sodu i kwasem solnym. Otrzymana sól diazoniowa reaguje z diselenkiem sodu. W wyniku tej reakcji uzyskiwany jest dimer kwasu 2,2'-diselenobisbenzoesowego. Następnie dimer poddawany jest reakcji z chlorkiem tionylu dając w efekcie chlorek 2-(chloroseleno)benzoesowy. W ostatnim etapie przeprowadzono sprzęganie obu fragmentów w środowisku zasadowym, otrzymując oczekiwane pochodne **24-26** (Schemat 9) [16].



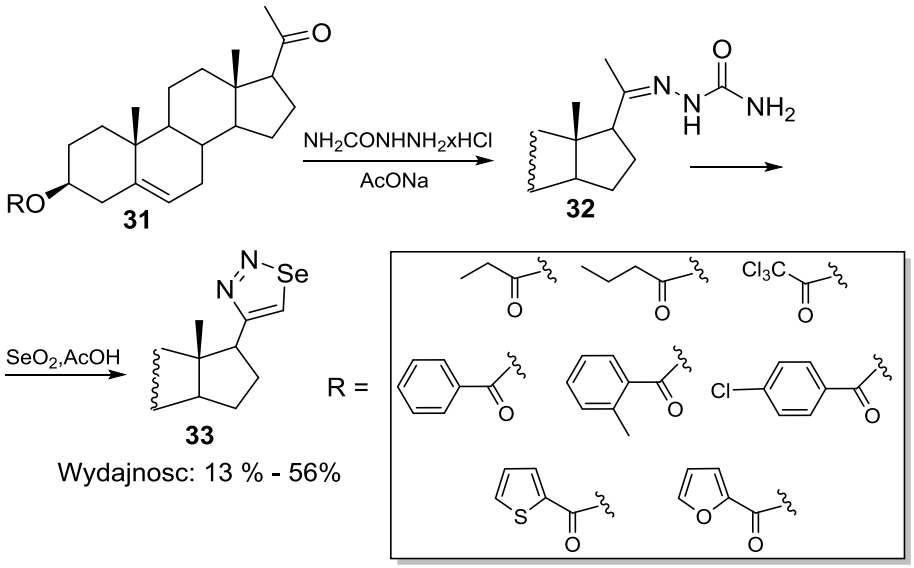
Schemat 9. Otrzymywanie steroidowych pochodnych ebselenu.

Opisano również syntezę steroidowych pochodnych ebselenu z wykorzystaniem dehydroepiandrosteronu. W pierwszym etapie za pomocą hydroksyloaminy w środowisku kwaśnym uzyskano oksym **27**. Otrzymany związek poddano redukcji z użyciem cyjanoborowodorku sodu i chlorku molibdenu(V) uzyskując aminosteroid **28**. Ostatnim etapem było sprzęganie uzyskanego związku z selenopochodną **29** (Schemat 10) [17].



Schemat 10. Otrzymywanie selenesteroidów na bazie dehydroepiandrosterionu.

Innymi selenosteroidami o właściwościach antyproliferacyjnych są pochodne pregnelonu. W pierwszym etapie substrat poddawany jest reakcji z semikarbazydem dając semikarbazon (**32**). Uzyskany związek został przekształcony do odpowiedniego selenodiazolu(**33**) z wykorzystaniem tlenku selenu(IV) i kwasu octowego. Uzyskano szereg pochodnych o właściwościach przeciwnowotworowych (Schemat 11) [18].

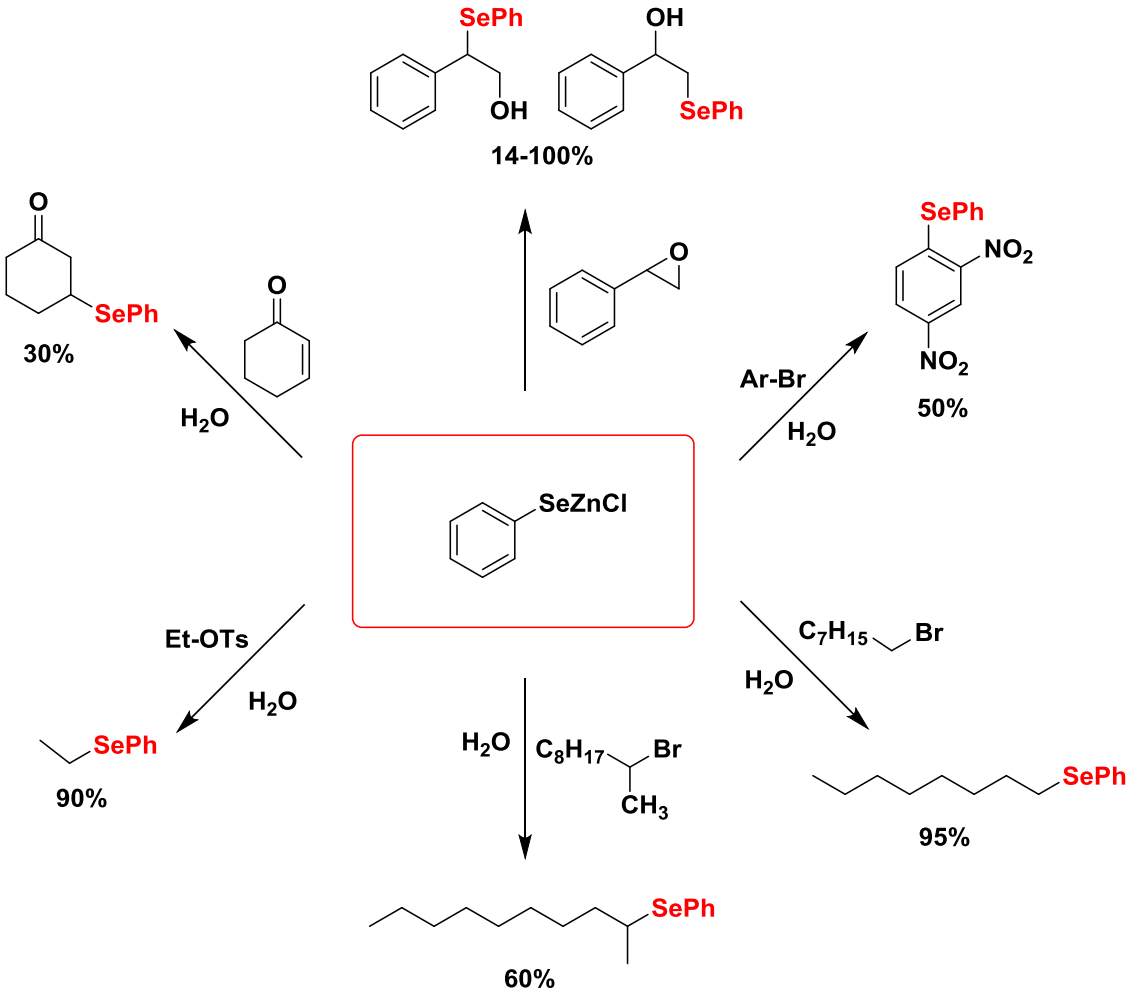


Schemat 11. Synteza selenosteroidów

Część literaturowa stanowi fragment pracy przeglądowej: I Jastrzebska, **P A. Grzes**, K. Niemirowicz-Laskowska, H Car, „Selenosteroids - promising hybrid compounds with pleiotropic biological activity: synthesis and biological aspects" *Journal of Steroid Biochemistry and Molecular Biology* 213 **(2021) ,** będącej publikacją składającą się na dysertację.

5. Omówienie wyników

W publikacji **P2** przedstawiłem metodę otrzymywania selenosteroidów za pomocą PhSeZnCl (tzw. reagent Santiego). Odczynnik ten w przeciwieństwo do innych nukleofilowych reagentów selenoorganicznych wykazuje się znacznie większą stabilnością. Reakcje można przeprowadzać w warunkach wodnych, reagent nie ulega gwałtownemu rozkładowi w kontakcie z tlenem. Charakteryzuje się szerokim spektrum reaktywności z różnymi związkami: epoksydami, tosylanami, związkami α,β-nienasyconymi oraz halogenkami alkilowymi i arylowymi (Schemat 12) [19].

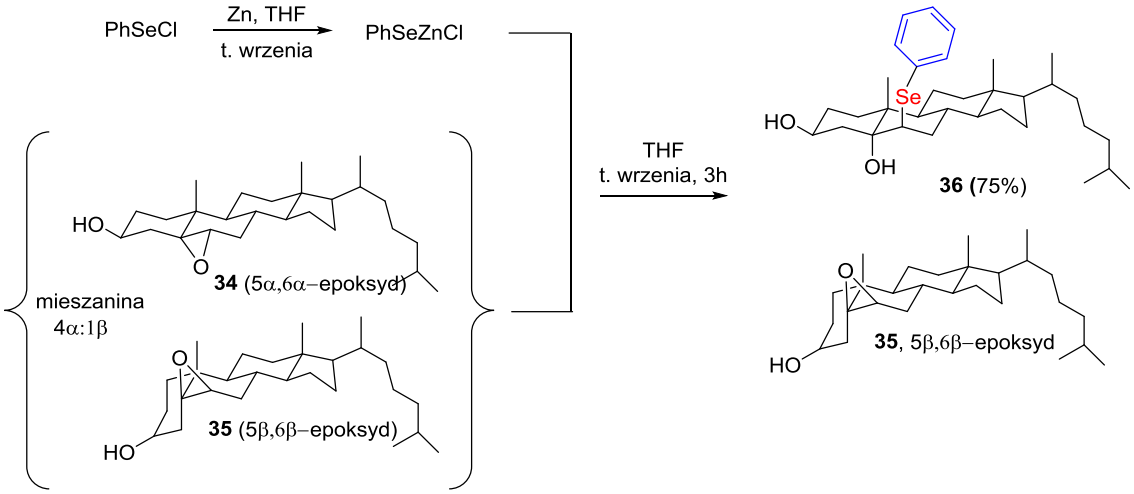


Schemat 12. Reaktywność PhSeZnCl z różnymi substratami. [19]

W swoich badaniach postanowiłem sprawdzić reaktywność tego odczynnika ze związkami steroidowymi. W tym celu otrzymałem szereg epoksydów steroidowych, poprzez reakcję z MCPBA w DCM (Tabela 1). Oprócz epoksydów zbadałem reaktywność innych pochodnych steroidowych z PhSeZnCl (Tabela 2).

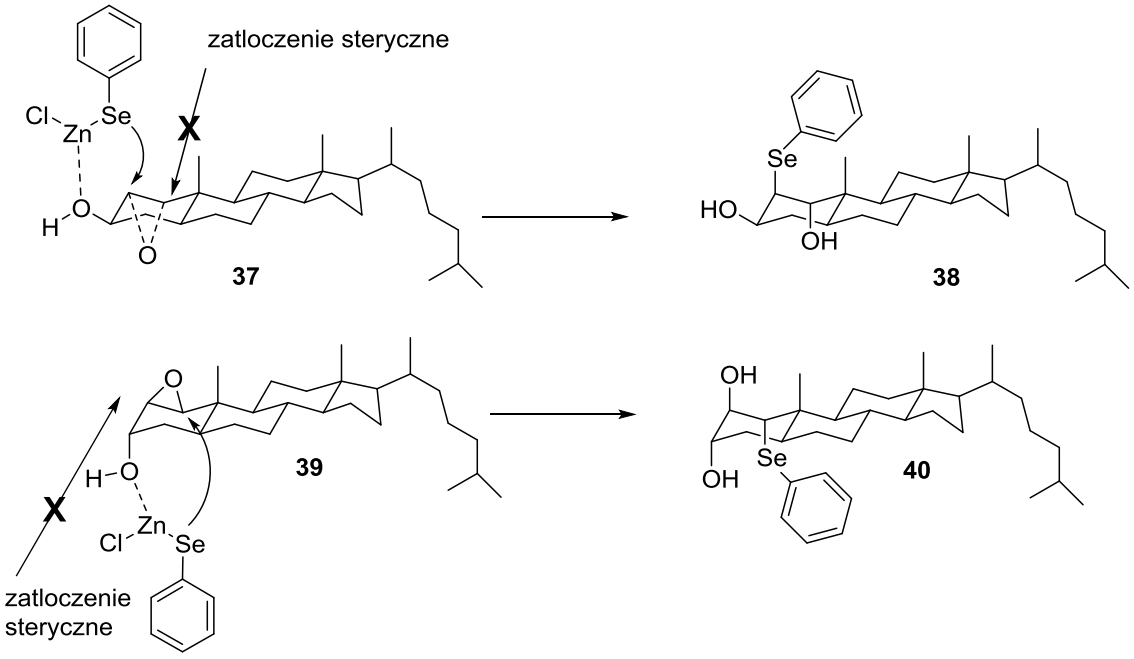
Pierwsze eksperymenty przeprowadzone były na mieszaninie izomerów 5,6-epoksycholestanu (**34, 35)**. Zastosowano mieszaninę izomerów α:β w stosunku 4:1 z wykorzystaniem PhSeZnCl w warunkach opisanych dla otwarcia epoksydu w zawiesinie wodnej oraz w THF w temperaturze pokojowej. Zastosowane warunki procesu nie przyniosły oczekiwanych rezultatów. Dopiero po wygenerowaniu odczynnika *in situ* i prowadzenie reakcji w temperaturze wrzenia, doprowadziło do otrzymania transhydroksyselenku **36**. Nieprzereagowany epoksyd **35** odzyskałem ilościowo metodą chromatograficzną (Schemat 13). Prawdopodobnie powodem braku

reaktywności β izomeru jest powstawanie niekorzystnej i niestabilnej struktury, która wynikałaby z obecności grupy hydroksylowej przy *C-5* zorientowanej w konfiguracji *syn* względem grupy metylenowej *C-19*.



Schemat 13. Reakcja otwierania epoksydu z odczynnikiem Santiego.

Schemat 14 obrazuje powstawanie wyłącznie izomeru **38,** co jest spowodowane obecnością aksjalnej grupy metylenowej w pozycji *C-19,* przez co atak nukleofilowy jest możliwy wyłącznie od mniej zatłoczonej strony epoksydu **37**.



Schemat 14. Prawdopodobny mechanizm otwierania epoksydów steroidowych z regentem Santiego.

Tabela 1 przedstawia uzyskane produkty z epoksysteroidów Reakcje prowadziłem w warunkach opracowanych dla epoksydu **34**. W niektórych przypadkach czas reakcji został wydłużony nawet do 6 godzin.

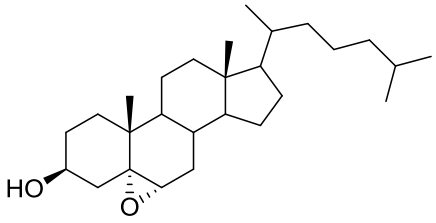
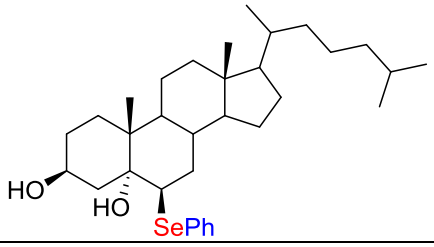
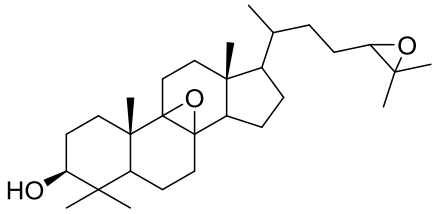
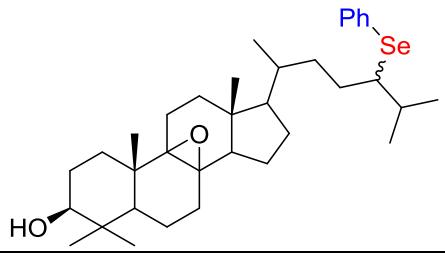
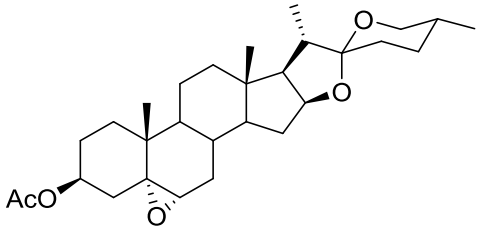
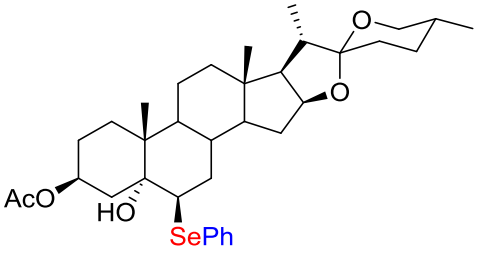
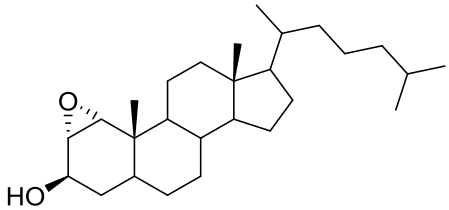
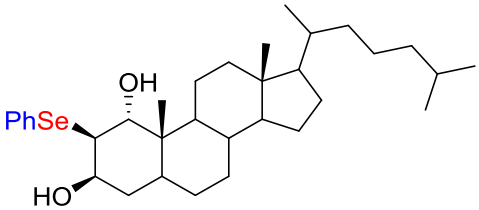
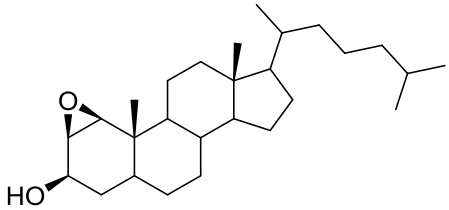
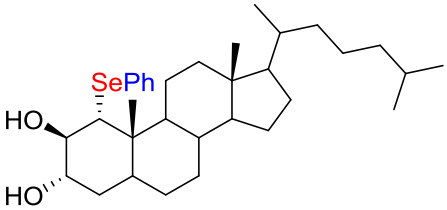
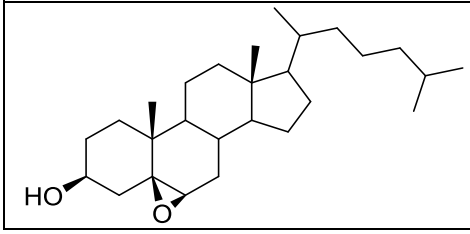
substrat	**Czas(h)**	**produkt**
	3	
	6	
	6	
	3	
	3	
	3	Brak reakcji

Tabela 1. Produkty reakcji otwierania epoksydów z reagentem Santiego.

Oprócz epoksydów steroidowych, reakcji poddałem inne pochodne steroidowe posiadające w swojej strukturze układ α,β nienasyconego ketonu, lakton oraz oksym (Tabela 2).

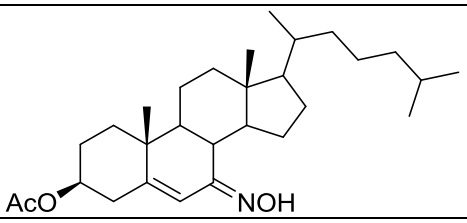
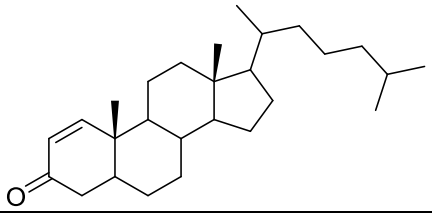
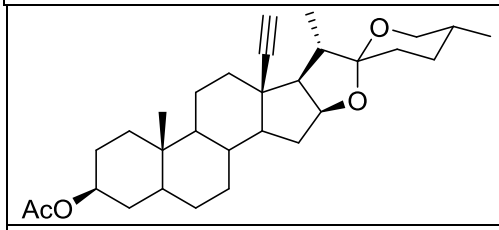
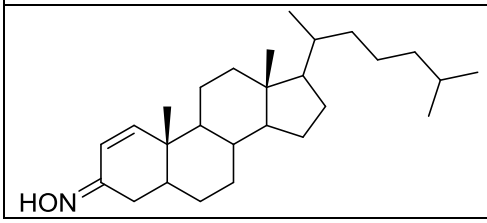
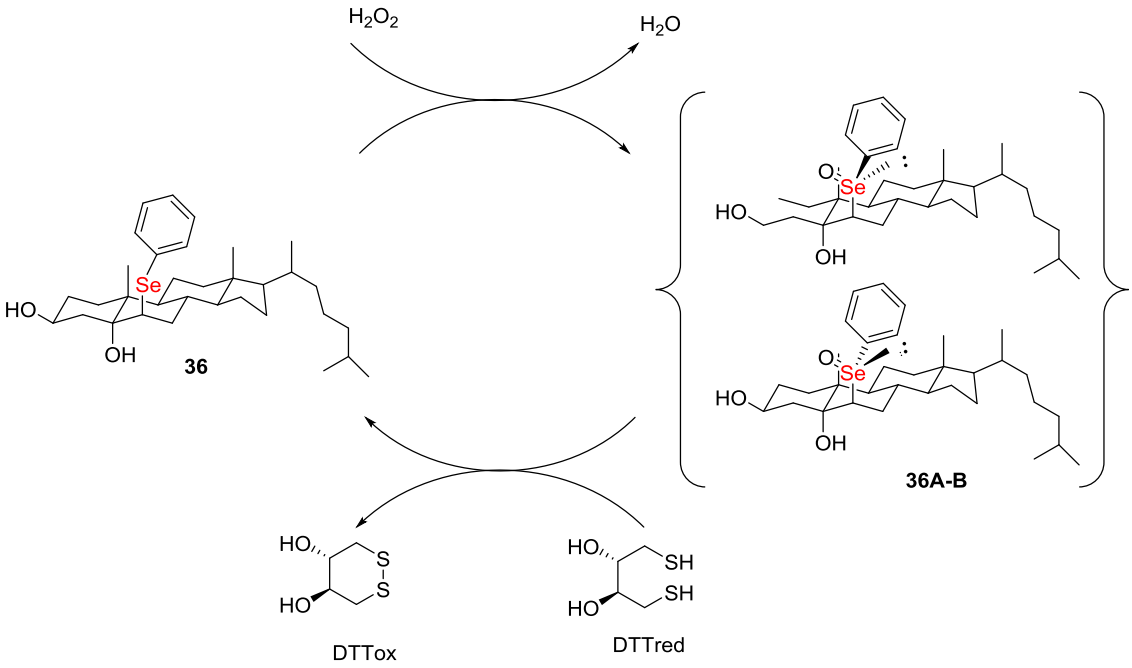
substrat	Czas(h)	produkt
	6	Brak reakcji
	6	Brak reakcji
	3	Brak reakcji
	3	Brak reakcji

Tabela 2. Reaktywność innych steroidów z PhSeZnCl.

Biorąc pod uwagę prooksydacyjne właściwości związków selenu, został przeprowadzony eksperyment sprawdzający właściwości redukcyjne otrzymanego selenosteroidu **36**. W tym celu została wykorzystana spektroskopia ^{1}H NMR i ^{77}Se NMR. Eksperyment polegał na dodaniu do próbki selenosteroidu **36** 5 ekwiwalentów nadtlenku wodoru i obserwacje zmian na widmie ^{77}SeNMR. Po całkowitej konwersji związku zaobserwowano na widmie powstanie pary diasteroizomerów selenotlenków **36A-B** CD_3OD: 901 i 866 ppm. Następnym etapem było dodanie stechiometrycznej ilości ditiotreitolu (DTT) i redukcja otrzymanych selenotlenków do substratu **36.** Kinetykę reakcji badano poprzez ^{1}H NMR. Proces okazał się bardzo powolny, ponieważ po 19 godzinach zaobserwowano, że jedynie 22% DTT uległo konwersji. Dla

porównania, wartość ta dla ebselenu wynosi 15 minut. [20] Powodem tak niskiej konwersji jest prawdopodobne silne zatłoczenie wokół atomu selenu (Schemat15).

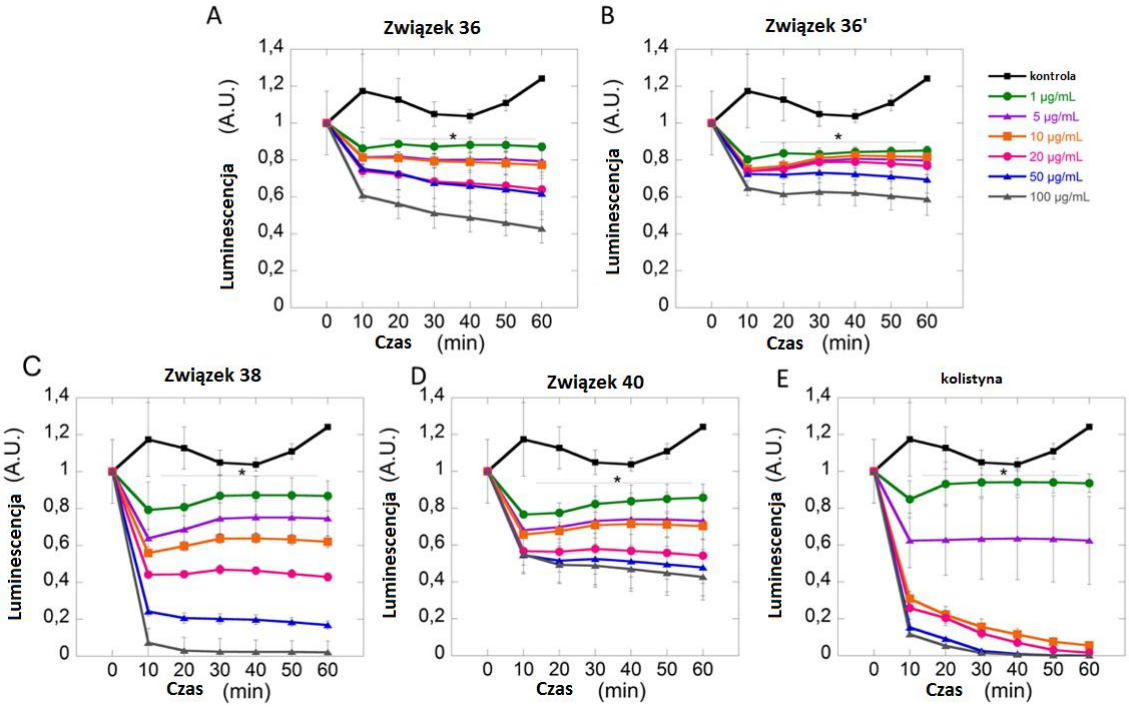


Schemat 15. Eksperyment sprawdzający właściwości redukcyjne otrzymanego selenosteroidu **36**.

Przeprowadzone badania biologiczne miały na celu ocenę właściwości przeciwbakteryjnych otrzymanych selenosteroidów. W obecnych czasach poszukiwanie związków o tych własnościach stanowi duże wyzwanie, zwłaszcza w kontekście rosnącej liczby zakażeń wywołanych przez antybiotykoodporne bakterie. Właściwości powodujące zaprzestanie tworzeniu biofilmu przez *Pseudomonas aeruginosa,* bakterii odpowiedzialnej za zakażenia u pacjentów z osłabionym układem odpornościowym, może być ważnym elementem rozwoju nowej klasy antybiotyków.[21]).

Pomiar zmian w luminescencji *Pseudomonas aeruginosa* jest łatwym narzędziem w ocenie żywotności bakterii i jej metabolizmu.[22]. Rysunek 1. pokazuje, że wszystkie przetestowane selenosteroidy wpływają na badany szczep. Najwyższą aktywność wykazywał związek **38** w porównaniu do pozostałych przebadanych związków. Zaobserwowano ponad 95% spadek chemiluminescencji po 10 minutach inkubacji przy najwyższej zadanej dawce związku. Ten efekt hamujący jest porównywalny do aktywności standardowych antybiotyków stosowanych w stężeniach odpowiadających 100-krotnemu wzrostowi wartości minimalnego stężenia hamującego (MIC). Uzyskane wyniki sugerują, że położenie grupy fenylselenylowej może wpływać

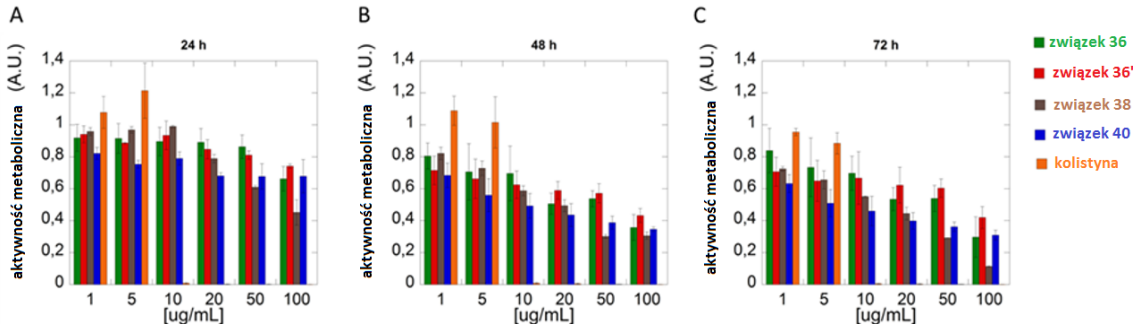
i modyfikować aktywność przeciwbakteryjną. Dodatkowo, aktywność syntetyzowanych związków porównano z aktywnością kolistyny, obecnie stosowanej w leczeniu zakażeń *P. aeruginosa*. Co ciekawe, zaobserwowano, że stosowanie kolistyny w dawce odpowiadającej 1xMIC nie wpływa na funkcję metaboliczną zakażeń *P. aeruginosa* wywołanych przez szczepy oporne na leki. Natomiast zastosowanie stężenia 5-krotnie większego od MIC zaburza funkcję komórek w stopniu 60%.



Rysunek 1. Obniżenie aktywności metabolicznej bakterii *Pseudomonas aeruginosa* przy użyciu otrzymanych selenosteroidów w porównaniu do kolistyny.

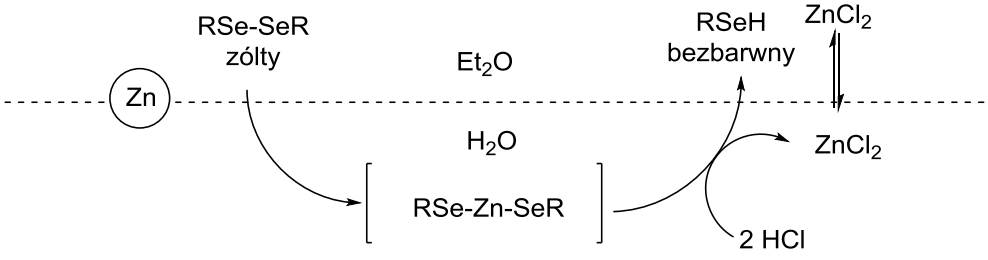
W ramach innych eksperymentów zastosowano pomiary luminometryczne w celu określenia zdolności przetestowanych selenosteroidów do zapobiegania tworzenia się bakteryjnego biofilmu. Przetestowane selenosteroidy są zdolne do hamowania tworzenia się biofilmu i skutecznego zabijania bakterii osadzonych w macierzy biofilmu w sposób zależny od czasu i dawki. Jednak po 24 godzinach, w przypadku związku **38**, konieczne było zastosowanie wysokich dawek (> 50 µg/ml), aby osiągnąć około 50% hamowania tworzenia się biofilmu. W przypadku dojrzałego biofilmu, utworzonego po 48 i 72 godzinach leczenia przetestowanymi substancjami (> 20 µg/ml), stwierdzono zmniejszenie żywotności biofilmu o około 50% dla związku **36** odpowiednio, oraz o około 90% dla związku **38.** W przypadku kolistyny, jej stosowanie w 1 i 5-krotnie większym

stężeniu minimalnego stężenia hamującego (MIC) było niewystarczające do ograniczenia metabolicznej aktywności biofilmu (Rysunek 2). Właściwości przetestowanych substancji mogą zatem pomóc w opracowaniu skutecznych strategii przeciwko tworzeniu się biofilmu *Pseudomonas aeruginosa*, który jest bezpośrednio związany z zakażeniami szpitalnymi poprzez kolonizację urządzeń medycznych, a także stanowi główną przyczynę nawrotów i przewlekłych zakażeń, takich jak zapalenie płuc u pacjentów z mukowiscydozą. Ze względu na amfipatyczną naturę przetestowanych steroidów oraz ich podobieństwo do ceragenin, które posiadają szerokie spektrum aktywności przeciwbakteryjnej, konieczne są dalsze badania [23].



Rysunek 2. Badanie aktywności otrzymanych selenosteroidów przed powstawaniem biofilmu bakterii *Pseudomonas aeruginosa* po 24, 48 i 72 godzinach.

W publikacji **P3** opisano syntezę selenosteroidów w oparciu o system dwufazowy opracowany przez Santi *et. A* oraz modyfikację tej metody syntezy, która polega na generowaniu nukleofilowych reagentów seleneorganicznych w układzie woda - rozpuszczalnik organiczny [24]. W pierwszym etapie diselenek poddawany jest reakcji z aktywowanym cynkiem dając przejściowy związek kompleksowy, który natychmiast reaguje z kwasem solnym generując bezbarwny selenol oraz chlorek cynku(II) (Rysunek 3).



Rysunek 3. System dwufazowy [24].

Metoda ta pozwala na funkcjonalizację wielu rodzajów substratów z bardzo dobrymi wydajnościami. System dwufazowy znalazł swoje zastosowanie w reakcjach z halogenkami, epoksydami oraz reakcji Michaela (Tabela 3) [24].

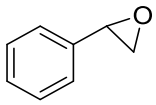
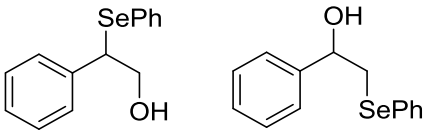
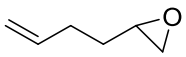
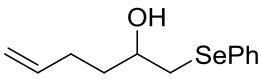
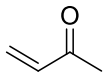
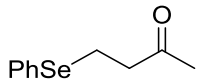
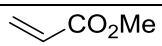
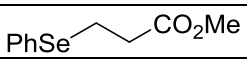
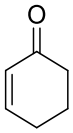
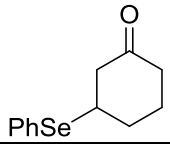
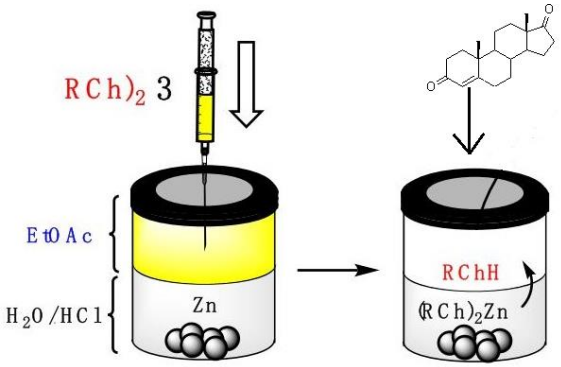
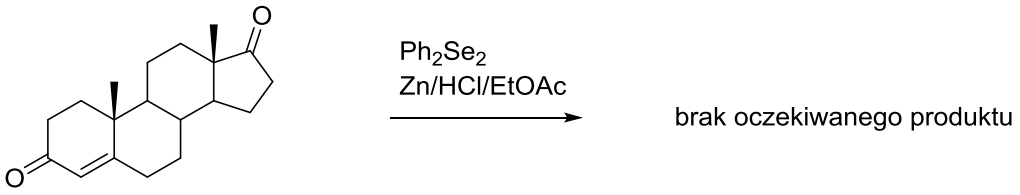
Substrat	Produkt	Czas (h)	Wydajność (%)
CH_3I	CH_3SePh	2	99
$CH_3(CH_2)_5Br$	$CH_3(CH_2)_5SePh$	72	85
	 19:1	4	78
		4	99
		2	91
		2	79
		2	88

Tabela 3. Reakcje wybranych substratów w systemie dwufazowym [24].

W swoich badaniach postanowiłem wykorzystać system dwufazowy do otrzymywania selenosteroidów w wyniku reakcji Michaela. Jako materiał testowy zastosowałem androstedion posiadający układ α,β-nienasyconego ketonu oraz diselenek difenylu jako reagent (Schemat 16). Pierwsze próby nie przyniosły oczekiwanych rezultatów.

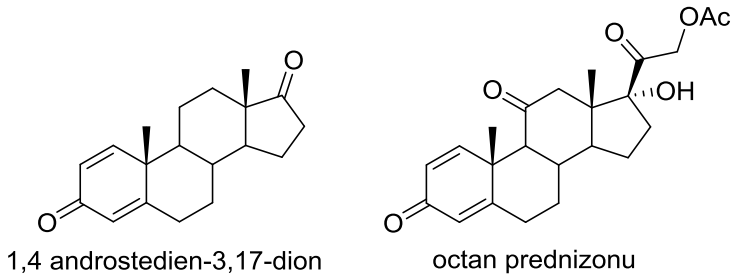


Rysunek. 3. Synteza selenosteoidów w system dwufazowym.



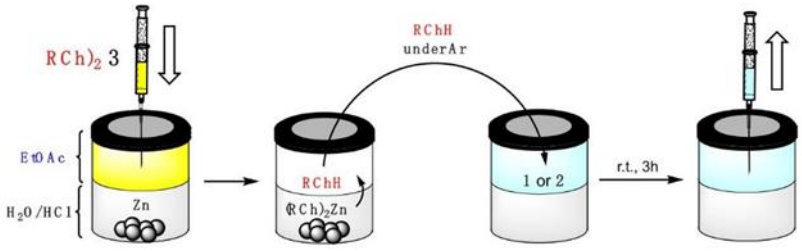
Schemat 16. Nieudana synteza selenosteroidu z androstedionem.

Przyczyną braku reakcji androstedionu z wygenerowanym selenolem jest prawdopodobnie zawada przestrzenna występująca pomiędzy pierścieniami A i B w szkielecie steroidowym. W związku z powyższym postanowiłem zmienić substrat na 1,4-androstedien-3,17-dion oraz octan prednizonu, ze względu na obecność dodatkowego wiązania podwójnego w pozycji *C1-C2*.(Rysunek 4).

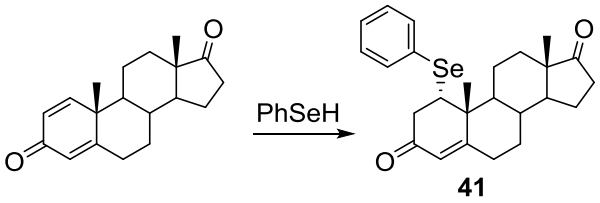


Rysunek 4.Struktury związków modelowych.

Brak oczekiwanego produktu wymusił modyfikację procedury w systemie dwufazowym. (Rysunek 5), która polegała na tym, aby po odbarwieniu roztworu przenieść roztwór bez cynku za pomocą kaniuli do kolbki zawierającej substrat w atmosferze gazu obojętnego. Zmiana spowodowała powstawanie oczekiwanego produktu **41** (Schemat 17).



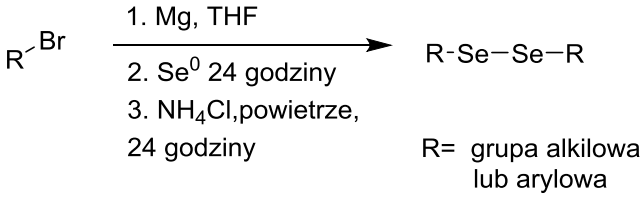
Rysunek 5. Modyfikacja procedury system dwufazowego.



41

Schemat 17. Synteza selenosteroidu w zmodyfikowanym układzie dwufazowym.

Przeprowadziłem identyczną reakcję z octanem prednizonu uzyskując oczekiwany selenosteroid **50**. Postanowiłem więc otrzymać serię pochodnych stosując różne diselenki. Jednak, handlowo dostępny był wyłącznie diselenek difenylu. Nowe diselenki otrzymałem poprzez reakcję wybranych halogenków z magnezem generując odczynnik Gringarda a następnie dodanie selenu elementarnego [25]. Po 24 godzinach w warunkach gazu obojętnego, dodawany jest nasycony roztwór chlorku amonu i roztwór miesza się przez kolejne 24 godziny w obecności tlenu. Surowy produkt oczyszczano poprzez krystalizację w etanolu lub chromatograficznie. (Schemat 19.)



Schemat 19. Synteza diselenków [25]

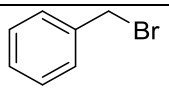
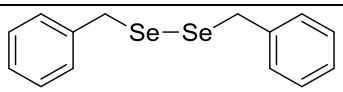
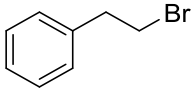
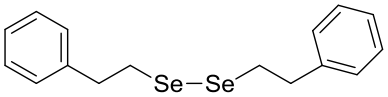
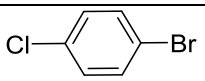
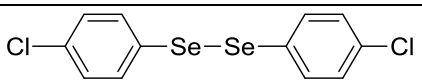
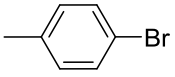
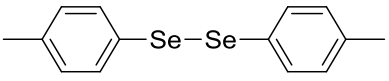
Halogenek	**Diselenek**	**Wydajność (%)**
	 **42**	**56**
	 **43**	**45**
	 **44 A**	**52**
	 **44B**	**55**

Tabela 4. Uzyskane diselenki

Uzyskane diselenki **42-44** Tabela 4) zostały wykorzystane do otrzymania nowych pochodnych z 1,4-androstedien-3,17-dionem. Wyniki zebrano w Tabeli 5.

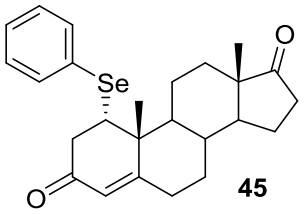
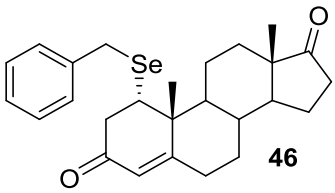
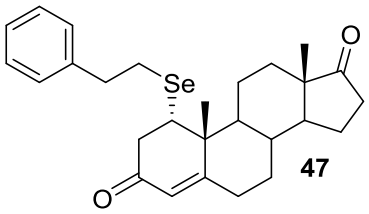
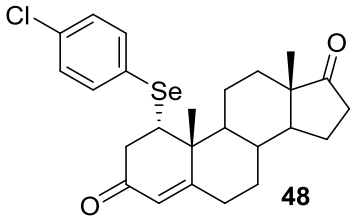
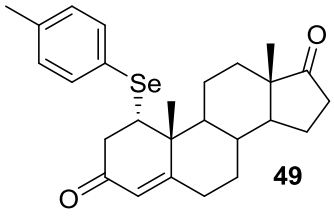
Diselenek	**Produkt**	**Wydajność (%)**
	 **45**	**70**
39	 **46**	**48**
40	 **47**	**51**
41	 **48**	**81**
42	 **49**	**64**

Tabela 5. Otrzymane selenosteroidy na bazie 1,4 androstedien-3,17-dionu.

Analogiczne reakcje przeprowadziłem przy użyciu drugiego substratu modelowego - octan prednizonu (Tabela 6).

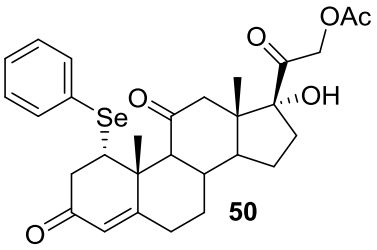
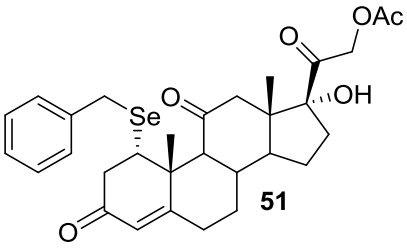
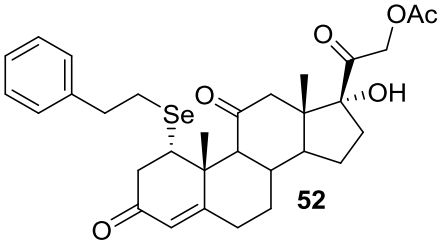
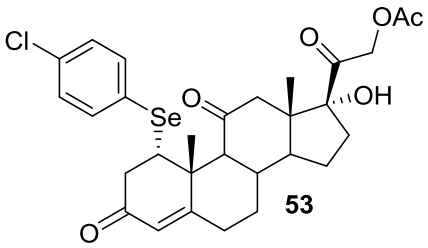
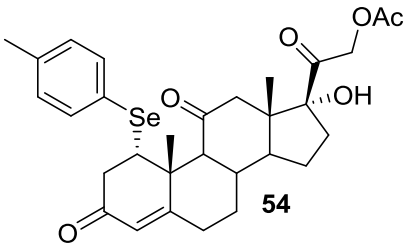
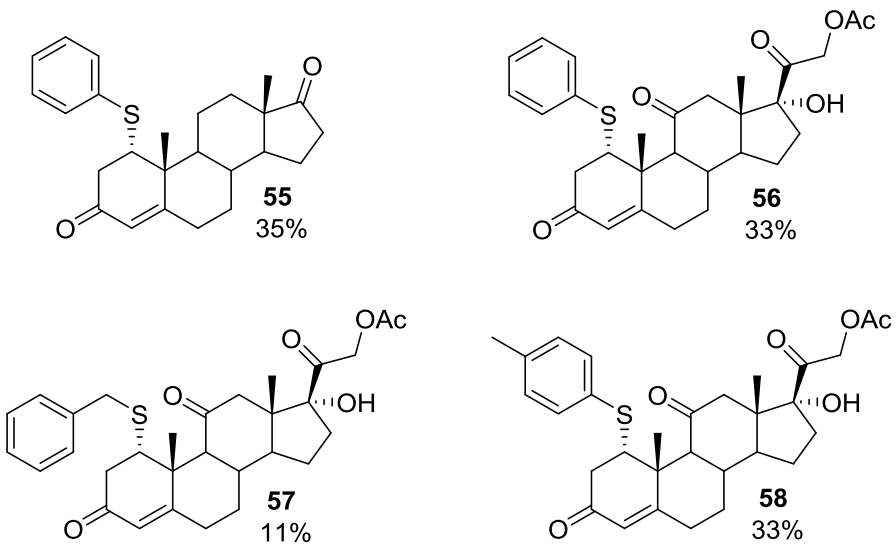
Diselenek	**Produkt**	**Wydajność %**
	 **50**	**54**
39	 **51**	**52**
40	 **52**	**46**
41	 **53**	**34**
42	 **54**	**96**

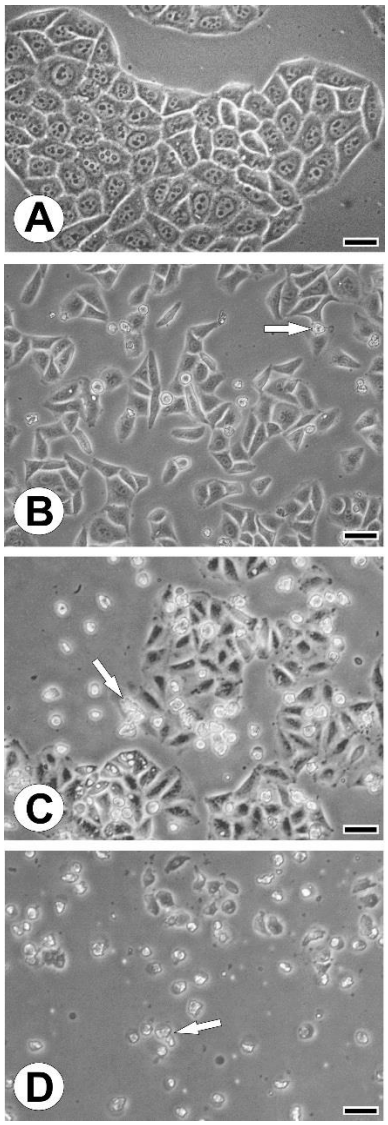
Tabela 6. Selenosteroidy otrzymane na bazie octanu prednizonu.

Zmodyfikowaną procedurę zastosowałem również do otrzymywania tiosteroidów **55-58** (Rysunek 6).. Wydłużono czas na generowanie tiolu z 10 minut do 60 minut



Rysunek 6. Otrzymane tiosteroidy w systemie dwufazowym.

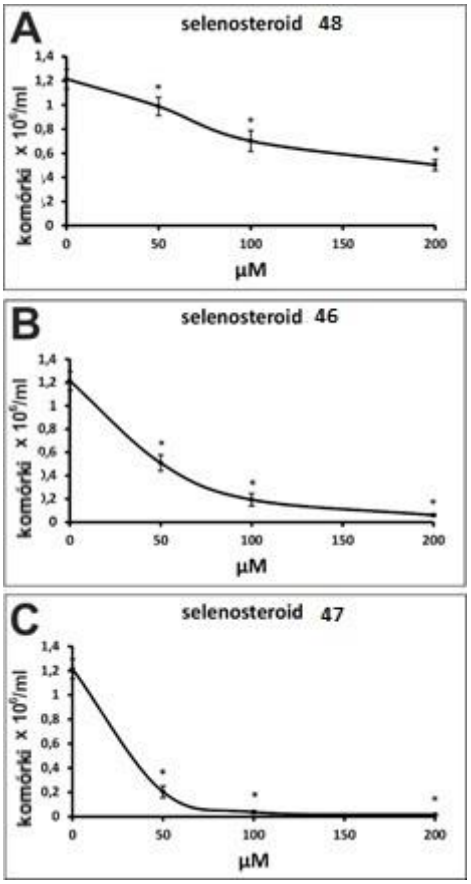
Przeprowadzone badania biologiczne miały na celu ocenę właściwości antyproliferacyjnych trzech selenosteroidów **46, 47, 48**. Wspomniane wcześniej związki zostały przebadane na linii komórkowej HeLa. Obserwacja mikroskopowa wykazała mniejszą liczbę oraz gorszy stan komórek hodowlanych z dodatkiem testowanych selenosteroidów. Po 48 godzinach eksperymentu pod wpływem badanych selenosteroidów zaobserwowano zmniejszoną liczbę komórek w kulturze w porównaniu z kontrolą. Najgorszy stan komórek zaobserwowano na pożywce ze związkiem **47**, gdzie odnotowano najmniejszą liczbę komórek przylegających do naczynia hodowlanego. (Rysunek 7).



Rysunek 7. Obserwacja mikroskopowa komórek Hela po 48h kontakcie z selenosteroidami w stężeniu 50 μM. Zdjęcia A- kontrola, B-hodowla komórek ze związkiem **48**, C- hodowla komórek ze związkiem **46**, D- hodowla komórek ze związkiem **47.** Strzałkami zaznaczono odklejające się od naczynia prawdopodobnie martwe lub apoptyczne komórki.

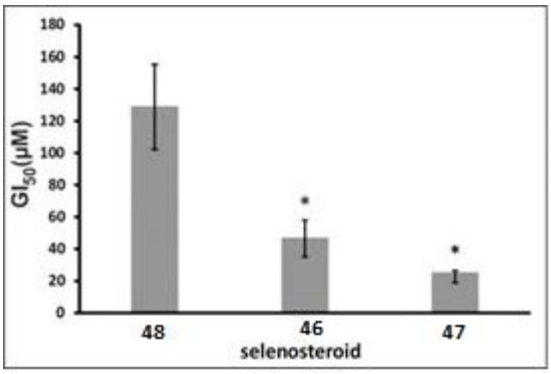
Została przeprowadzona szczegółowa analiza liczby komórek w kulturach po trzech dniach eksperymentu, gdy kultura kontrolna osiągnęła około 90% konfluencji. Uzyskane wyniki potwierdziły istotne statystycznie i zależne od stężenia hamowanie tempa wzrostu w hodowlach z dodatkiem badanych selenosteroidów w zakresie stężeń od 50 do 200 μM. Największą aktywność inhibicyjną wykazał związek **47**, gdzie przy stężeniu 50 μM(wykres **C**, Rysunek 8) zaobserwowano jedynie 15,6% komórek w

stosunku do kultury kontrolnej a stężenia 100 i 200 μM prawie całkowicie zahamowały przyrost liczby komórek. Mniejszy wpływ na tempo wzrostu kultury zaobserwowano pod wpływem związku **46,** przy 50 μM odnotowano 39,8%, a przy 100 μM 14,8% liczby komórek w porównaniu z kontrolą, a przy 200 μM niemal całkowite zahamowanie rozwoju kultury (wykres **B**, Rysunek 8). Najsłabszą aktywność antyproliferacyjną wykazywał związek **49**, gdzie przy stężeniu 200 μM jedynie 39% stwierdzono 39% liczby komórek w porównaniu do kontroli. (wykres **A**, Rysunek 8).



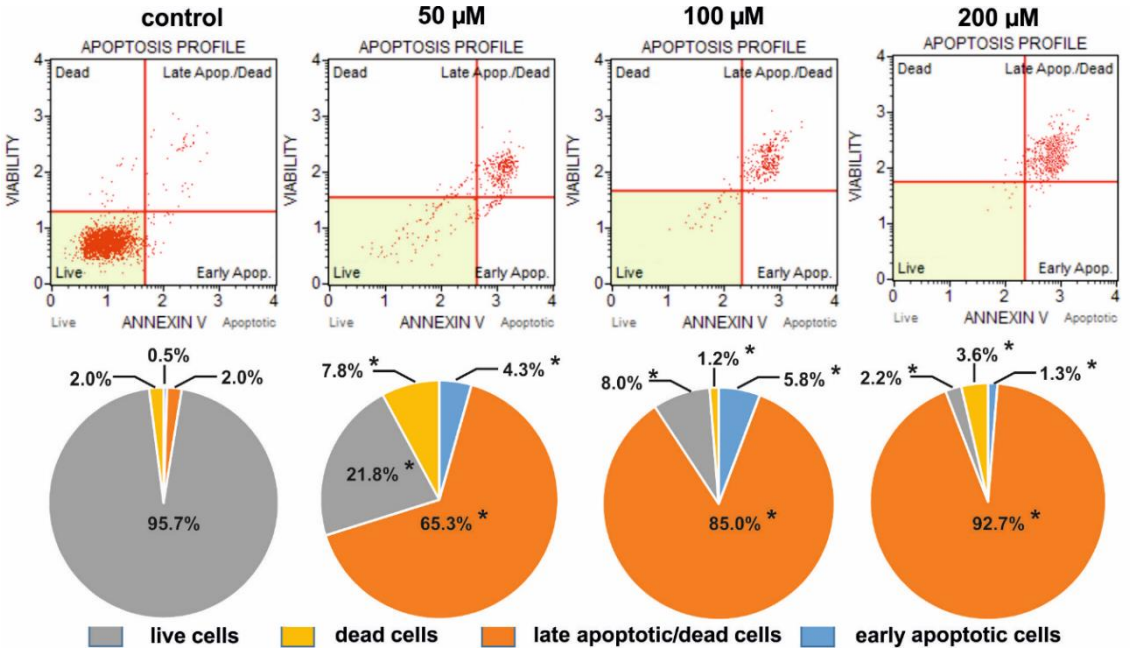
Rysunek 8. Zmiana liczby hodowlanych komórek HeLa w funkcji stężenia selenosteroidów po 3 dniach działania.

Na podstawie danych dotyczących zależności tempa wzrostu komórek od stężenia selenosteroidu, został wyliczony parametr GI_{50} dla każdego związku (Rysunek 9). Wartości te wynoszą odpowiednio 25,5 μM dla **48**, 47,2 μM dla **47** i 129,2 μM dla **49**.



Rysunek 9. Porównanie wartości GI_{50} badanych selenosteroidów, ich mediana. Gwiazdką oznaczono różnicę istotną statystycznie względem pochodnej **46.**

W celu zbadania mechanizmu działania badanych związków, przeanalizowano profil przeżywalności komórek oraz apoptozy pod wpływem selenosteroidów. Wykonana analiza cytometryczna potwierdziła proapoptotyczne właściwości badanych selenosteroidów. Najsilniejszą indukcję procesu apoptozy odnotowano w przypadku pochodnej **47** (Rysunek 10).



Rysunek 10. Porównanie profili apoptycznych hodowli kontrolnych komórek. HeLa oraz w różnych stężeniach selenosteroidu **47**. Histogramy przedstawiają analizę cytometryczną przykładowych. Diagramy kołowe przedstawiają procentowy rozkład poszczególnych rodzajów komórek, zestawiony z niezależnych eksperymentów. Gwiazdkami zaznaczono wartości istotnie różniące się względem kontroli.

W literaturze jest mało informacji dotyczących właściwości proapoptycznych selenosteroidów. Takie właściwości wykazywała pochodna diosgeniny posiadająca ugrupowanie N-fenyloselenomocznikowe w pozycji *C3* [26]. W tych badaniach stwierdzono około 60% komórek apoptycznych przy stężeniu 25 μM. Co ciekawe autorzy nie wykazali takich samych właściwości propaptycznych dla tiomocznikowej pochodnej diosgeniny. Selenosteroidy o takich samych właściwościach opisuje również w swych pracach Huang Y. *et al* [27,28,29]. W naszych badaniach selenosteroid **48** w stężeniu 50 μM powodował apoptozę około 70% komórek w hodowli.

Hodowle *in vitro* komórek HeLa z testowanymi związkami umożliwiły porównanie względnej ekspresji genów indukujących apoptozę z genami związanymi z syntezą cholesterolu. Spośród testowanych związków, indukcję ekspresji genów związanych ze syntezą cholesterolu (HMGCR, SQLE, CYP51A1) zaobserwowano dla związków **46** i **48** w odróżnieniu od związku **47**. Natomiast ekspresja genu PDHB była istotnie zmniejszona w kulturze na pożywce ze związkiem **47**, podczas gdy pozostałe związki nie wpłynęły na ekspresje tego genu. Obecność związku **48** w pożywce zwiększyła ekspresję proapoptotycznego genu BID w komórkach HeLa w porównaniu do komórek hodowlanych w obecności związków **46** i **47**, gdzie poziom tego transkryptu nie zmienił się istotnie w porównaniu z kontrolą. Jednak w przypadku genu APAF1, którego produkt stanowi istotny składnik apoptosomu [30], stwierdzono zmniejszenie ekspresji pod wpływem związków **46** i **48** oraz tendencję do ograniczenia jego ekspresji w przypadku związku **47.**

Uzyskane wyniki sugerują różne mechanizmy działania badanych selenosteroidów. Związki **46** i **48 ,** które słabiej hamują wzrost HeLa, działają w podobny sposób, podczas gdy związek **47** może mieć dodatkowe mechanizny działania.

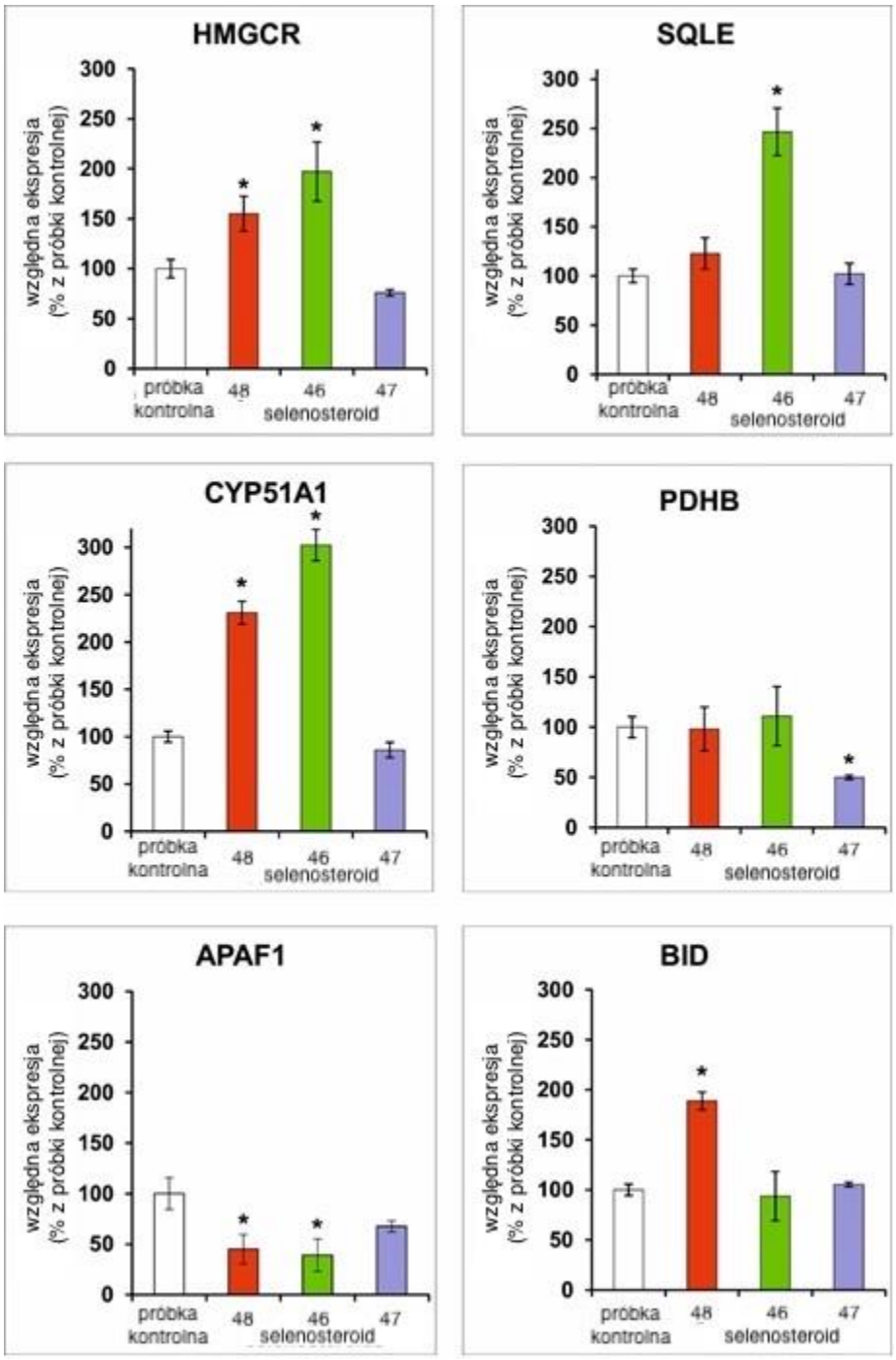
Wzrost ekspresji genów HMGCR, SQLE i CYP51A1 w próbkach zawierających związki **46** i **48,** wpływa na tempo szlaku mewalonowego, związanego z syntezą cholesterolu. Odpowiedź komórek polegająca na indukcji szlaku mewalonowego może prowadzić do wzrostu stężenia cholesterolu w błonie, co może chronić komórki przed jej destabilizacją. Obserwowany wzrost ekspresji tych enzymów pośrednio potwierdza hipotezę, że selenosteroidy jako antymetabolity cholesterolu prowadzą do destabilizacji błony komórkowej. Kluczowa rola szlaku mewalonowego jest znana podczas rozwoju nowotworu.[31,32]. Aktywacja genów odpowiedzialnych za biosyntezę cholesterolu

jest cechą charakterystyczna wielu rodzajów nowotworów chroniąc komórki nowotworowe przed działaniem leków i układu odpornościowego na zasadzie stabilizacji błony komórkowej.

Destabilizacja błony komórkowej spowodowana przez selenosteroidy może nieprawidłowo aktywować receptory zewnątrzpochodnego szlaku apoptozy co sugeruje zmieniona ekspresja genu BID. Białko BID jest aktywowane przez kapsazę 8, która jest proteazą inicjatorową zewnątrzpochodnego szlaku apoptozy. Obserwowane zmniejszenie ekspresji genu APAF1, którego produkt jest kluczowym czynnikiem apoptosomu, może być reakcją obronną komórek nowotworowych mającą na celu ograniczenie aktywacji Kaspar wewnątrzpochodnego szlaku apoptozy [33].

Biorąc pod uwagę powyżej przedstawiony mechanizm, można przyjąć, że działanie cytostatyczne badanych związków wynika z ich oddziaływania na błonę komórkową i indukcji zewnątrzpochodnego szlaku apoptozy.

Związek **47** w przeprowadzonym eksperymencie nie wpływa bezpośrednio na ekspresję genów związanych ze szlakiem mawalonowym oraz proapoptycznymi genami APAF1 i BID. Biorąc pod uwagę jego najsilniejsze działanie cytostatyczne, można wyciągnąć wniosek, że komórki mają czasu na aktywację mechanizmów obronnych, jak w przypadku mniej aktywnych pochodnych **46** i **48**. Należy zauważyć, że ekspresja genu PDHB jest ograniczona pod wpływem pochodnej **47**. Białko kodowane przez PDHB stanowi kluczowy składnik kompleksu dehydrogenazy pirogronianowej, która katalizuje oksydacyjną dekarboksylację pirogronianu do acetylo-CoA, który jest kluczowym metabolitem wielu procesów katabolicznych jak np. cykl Krebsa jak i anabolicznych jak synteza kwasów tłuszczowych i steroidów w komórkach [34]. Zahamowanie tej reakcji prowadzi do obniżenia zawartości ATP, redukcji syntezy lipidów co dalej przyczynia się do dezorganizacji błony komórkowej. Ograniczenie tych procesów może być głównym powodem najmocniejszych właściwości antyproliferacyjnych związku **48 (**Rysunek 11).



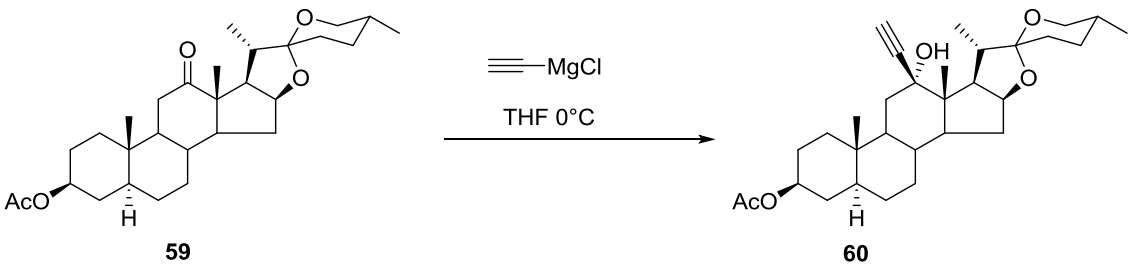
Rysunek 11. Porównanie względnej ekspresji wybranych genów po 3 dniach ekspozycji kultur HeLa na badane selenosteroidy.

Podsumowując wyniki wstępnych badań biologicznych i analizę dostępnych danych można stwierdzić iż badane selenosteroidy szczególnie **47** posiadają właściwości cytostatyczne w porównaniu do leków stosowanych w praktyce klinicznej takimi jak abirateron [35,36] czy doksorubicyna [37,38]. Wyniki te zachęcają do dalszych badań.

Opisane powyżej badania biologiczne stanowią fragment publikacji: I. Jastrzebska, N. Wawrusiewicz-Kurylonek, P. A. Grześ, E. Grabowska, A.Tylicki, „*Steroidal selenides as proapoptotic factors: a preliminary study"* przesłanej do recenzji.

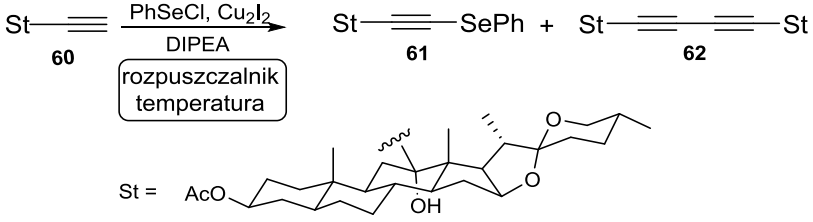
W publikacji **P4** przedstawiłem metodę otrzymywania selenosteroidów w wyniku sprzęgania terminalnych alkinów steroidowych z elektrofilowymi reagentami selenowymi z zastosowaniem katalizatorów miedzi(I). Warunki reakcji zoptymalizowano.

Substratem modelowym był 3β-octan-12β-etynylo-25R-5α-spirostan-3β,12α-diol **60** otrzymany w wyniku reakcji octanu hekogeniny **59** z odpowiednim odczynnikiem Gringarda (Schemat 20).



Schemat 20. Otrzymywanie substratu modelowego.

W celu uzyskania najwyższej wydajności przeprowadzanych reakcji przebadałem wpływ rozpuszczalników, temperatury i katalizatorów. Pierwsze testy miały na celu wybór odpowiedniego rozpuszczalnika oraz temperatury reakcji. Wyniki zestawiłem w Tabeli 7.



Schemat 21. Sprzęganie steroidowego alkinu z PhSeCl.

Rozpusczalnik	Temperatura	Wydajność 62 %	Wydajność 63 %
DCM	RT	-	-
DCM	R	-	-
THF	RT	-	-
Toluen	RT	-	-
Toluen	R	-	-
DMF	RT	-	-
THF	R	16%	25%
DMF	100°C	83%	-

Tabela 7. Wpływ rozpuszczalnika i temperatury na wydajność sprzęgania

Analizując wyniki z powyższej tabeli można zauważyć, że reakcja nie zachodzi gdy używany jest rozpuszczalnik niepolarny (toluen i dichlorometan), czy to w temperaturze pokojowej czy podczas podgrzewania. Użycie tetrahydrofuranu prowadzi do tworzenia produktu sprzęgania Glasera. Najlepsze rezultaty przyniosła reakcja z DMF w temperaturze 100°C. Warto nadmienić, iż warunki reakcji były kompatybilne z wrażliwym układem spiroacetalowym i nie obserwowałem powstawania dodatkowych produktów ubocznych.

W drugim etapie optymalizacji warunków reakcji testowałem dostępne katalizatory miedziowe. Wyniki zestawiono w Tabeli 8. Najlepsze rezultaty uzyskałem stosując Cu_2I_2.

katalizator	Wydajność (%)
Cu_2I_2	83
CuO	0
$CuSO_4$	0
CuOAc	0
CuCl	27
$FeCl_2$	0
CuI	16

Tabela 8 . Wpływ katalizatora miedziowego na wydajność reakcji

Po dokonaniu optymalizacji warunków procesu, opracowana metodologia została zastosowana dla różnych alkinów steroidowych **60**, **63**, **65** i **67** (otrzymanych przeze mnie w ramach projektu) uzyskując oczekiwane selenosteroidy (Tabela 9).

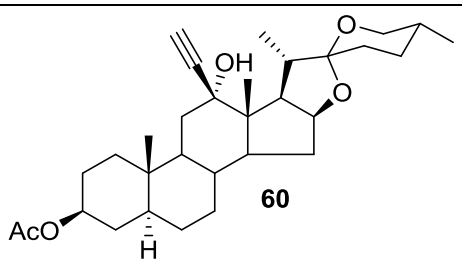
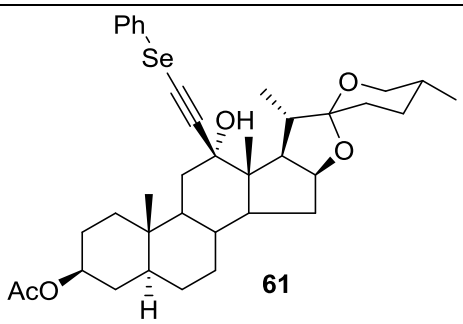
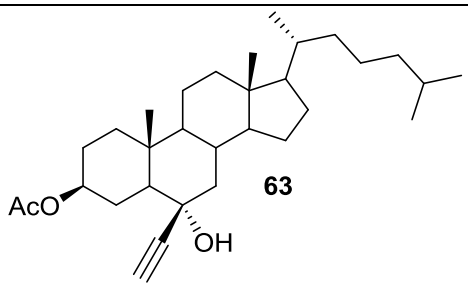
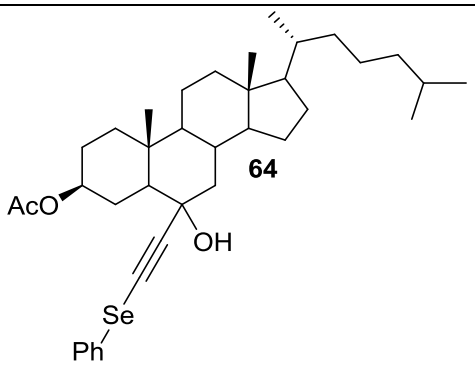
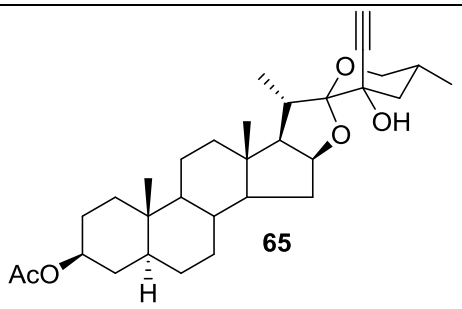
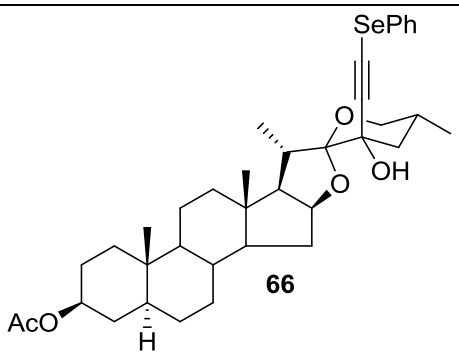
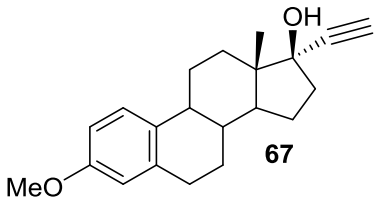
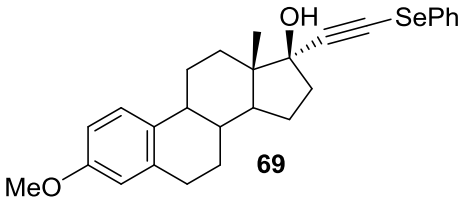
Substrat	**Produkt**	**Wydajność%**
 **60**	 **61**	83%
 **63**	 **64**	64%
 **65**	 **66**	96%
 **67**	 **69**	63%

Tabela 9. Otrzymane selenosteroidy w wyniku sprzęgania PhSeCl z alkinami steroidowymi.

Otrzymane pochodne przebadano pod kątem ich właściwości biologicznych na Uniwersytecie Medycznym w Białymstoku w zakładzie Farmakologii Doświadczalnej. Celem tych badań była ocena właściwości przeciwnowotworowych raka piersi. Na podstawie danych WHO z 2020 rak piersi został zdiagnozowany u ponad 2,3 miliona kobiet i zanotowano ponad 680 000 zgonów z tego powodu [39, 40].

Otrzymane selenosteroidy zostały poddane wielu testem mającym na celu oceny ich właściwości przeciwnowotworowych. Pierwszym z przeprowadzonych testów była ocena hemolizy czyli zdolności do niszczenia zdrowych komórek gospodarza. Wiele potencjalnych leków jest odrzucanych z powodu potencjalnych zniszczeń zdrowych komórek gospodarza. W przeprowadzonym eksperymencie użyto izolowanych czerwonych ludzkich krwinek jako modelu badawczego, umożliwiających ocenę potencjalnych zniszczeń w organizmie ludzkim. Jak przedstawiono na wykresie 2A (Rysunek 12) otrzymane związki **61**, **65**, **66** i **69** charakteryzują się niską aktywnością hemolityczną przy dawce 100 µg/ml. Testowane związki wykazywały hemolizę między 1% a 2,5%, co jest poniżej międzynarodowego standardowego poziomu 10%, który jest proponowany dla leków [41, 42]. Na podstawie uzyskanych wyników potwierdzono, że wszystkie zsyntetyzowane selenki steroidowe mają dobrą hemokompatybilność i będą odpowiednie do stosowania w podawaniu dożylnym.

Jednym z niepożądanych skutków prowadzonej chemioterapii jest negatywny wpływ na funkcjonowanie serca. Ustalono, że śmiertelność pacjentów z rakiem piersi związana jest z sercem, która jest spowodowana terapią antracyklinami, terapią hormonalną oraz inhibitorami kinazy tyrozynowej. Wspomniane leczenie może doprowadzić do niewydolności serca lub powstawania zakrzepów [43]. W celu oceny kardiotoksyczności otrzymane związki zostały poddane interakcji z linią komórkową H9C2, będących modelem w tego typu badań. Wyniki przedstawione na wykresie B (Rysunek 12) wskazują, że wszystkie badane substancje nie wykazywały istotnej zmiany liczby komórek w hodowli. Natomiast testy proliferacji i aktywności metabolicznej badanej linii komórkowej, wskazała , że związek **69** w stężeniu µg·mL powoduje statystycznie zahamowanie proliferacji komórek o 70% (wykres C Rysunek 12).

W celu określenia właściwości hamujących wzrost komórek, kultury zostały poddane działaniu związkami **61**, **65**, **66** i **69** w zakresie stężeń od 0 – 100 µg·mL− 1 przez 24 godziny a następnie przeprowadzono badania dotyczące cytotoksyczności i zdolności do proliferacji. Wykres D (Rysunek 12) przedstawia istotny efekt hamujący

testowanych związków w porównaniu do kontroli. Uzyskane wyniki pokazują ,że związki **65**, **66** i **69** w stężeniu 10 μg·mL− 1 hamowały żywotność komórek o połowę. Zwiększenie stężenia dla wszystkich związków wywoływało efekt cytotoksyczny dla wszystkich badanych selenosteroidów. Najsilniejszy efekt cytotoksyczny miały związki **66** i **69.**

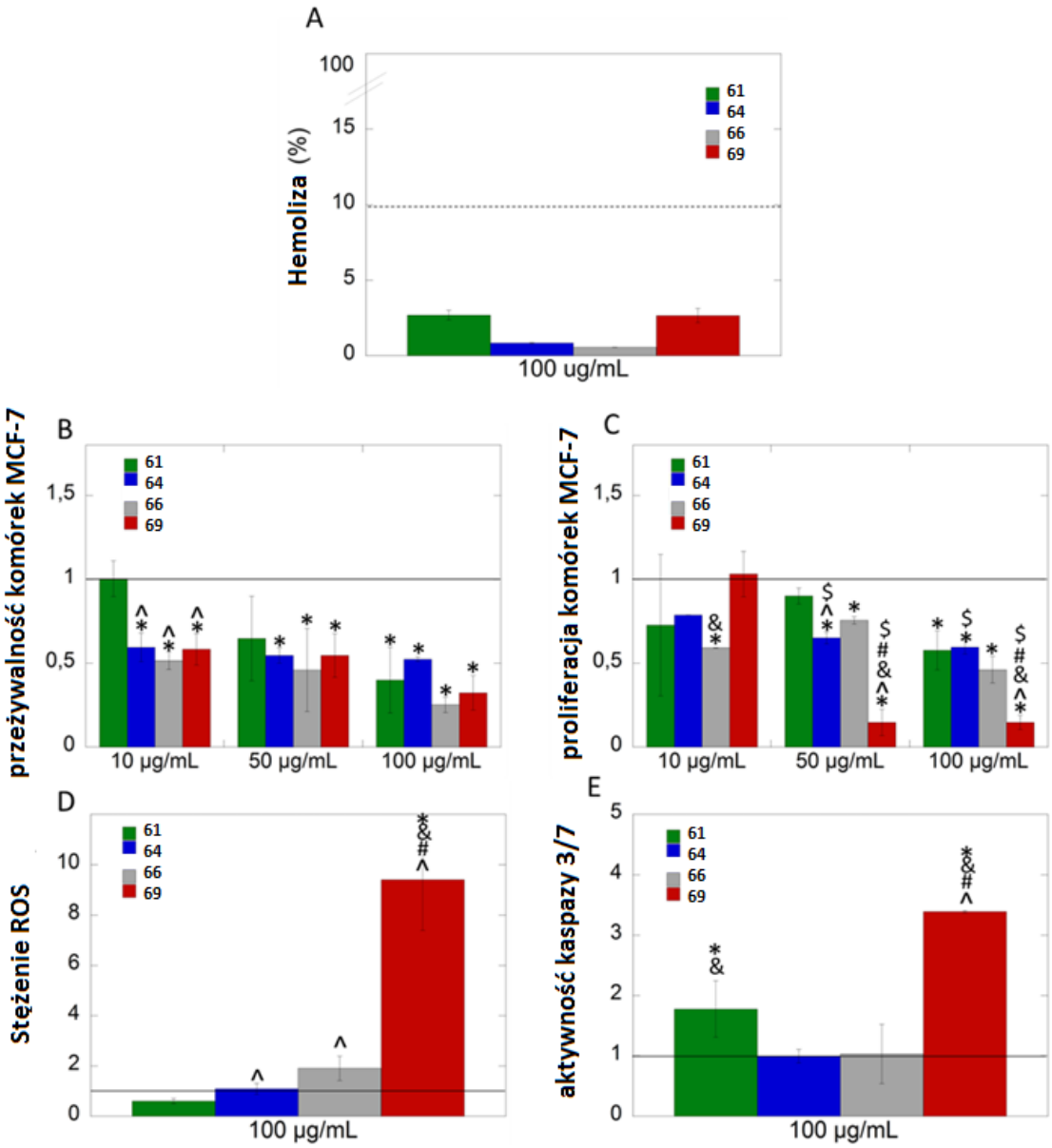
W dalszych badaniach postanowiono sprawdzić czy badane związki zaburzają równowagę utleniania i redukcji w badanych hodowlach. Zostały zbadane parametry związane ze stresem oksydacyjnym w tym ROS. Do przeprowadzania tych testów, przeprowadzono pomiary oparte na luminometrii [44]. Na wykresie F (Rysunek 12) tylko selenosteroid **69** spowodował znaczny wzrost stężenia ROS. Ilość wolnych rodników przy stężeniu 100 μg·mL− 1 spodował 10-krotny przyrost w porównaniu do próby kontrolnej.

Aby lepiej poznać mechanizm odpowiedzialny za śmierć komórki przeprowadziliśmy test aktywności kaspazy 3/7, białka zaangażowanego w proces apoptozy. Na wykresie G (Rysunek 12) możemy zaobserwować, że przy hodowlach linii MCF-7 maksymalna aktywność kaspazy obserwowana jest dla związku **69**. Można przyjąć, że mechanizm śmierci komórki jest związany z aktywnością kaspaz jest zależna od ilości generowanego ROS.

Aby lepiej określić potencjał selenosteroidów z obecnymi lekami obliczono wartość IC_{50} dla badanych związków oraz doksorubicyny (DOX). Wyniki zestawiono | w Tabeli 10.

Pochodna	**IC 50 [μM]**	
	MCF-7	**H9c2**
61	>100	>100
64	>100	>100
66	54 ± 18	>100
69	77 ± 17	>100
DOX	49 ± 2,35	5,5 ± 1,90

Tabela 10. Wartości IC_{50} badanych związków.



Rysunek 12.

6. Podsumowanie i wnioski

Niniejsza rozprawa doktorska prezentuje wyniki badań nad syntezą i właściwościami biologicznymi otrzymanych selenosteroidów.

Powyższe związki otrzymano według procedur opisanych w literaturze. Wymagały one jednak modyfikacji ze względu na specyficzną strukturę, sztywność układu oraz obecność reaktywnych grup funkcyjnych steroidów.

Procedura otrzymania selenosteroidów z regentem Santiego wymagały sporej modyfikacji względem metody opisanej w literaturze. Reakcje musiały być prowadzone w temperaturze wrzenia rozpuszczalnika, a odczynnik był otrzymywany *in situ*. Pomimo zastosowanych warunków prowadzonych syntez, PhSeZnCl znalazł jedynie zastosowanie w otwieraniu epoksysteroidów, lecz nie innych pochodnych steroidowych.

Uzyskane związki **36**, **36'**, **38** i **40** przebadano pod kątem ich właściwości antybakteryjnych. Związek **38** wykazywał silne właściwości zapobiegające powstawaniu biofilmu antybiotykoopornej bakterii *Pseudomonas aeruginosa* oraz hamujących metabolizm tej bakterii.

Metodologia uzyskania selenosteroidów **45 - 54** z wykorzystaniem systemu dwufazowego, również wymagała modyfikacji względem oryginalnej procedury. Udało się otrzymać serię nowych selenosteroidów z dobrymi wydajnościami. Problemem okazała się niestabilność niektórych pochodnych, zwłaszcza opartych na octanie prednizonu. Związki te ulegały rozkładowi po kilkugodzinnej ekspozycji na światło i warunki atmosferyczne.

Selenosteroidy **46-48** zostały przebadane pod kątem właściwości cytostatycznych. Przebadano wpływ tych związków na zachowanie linii komórkowej HeLa. Wszystkie związki wykazały właściwości proapoptyczne i doprowadzały do destabilizacji błony komórkowej. Badania ekspresji genów odpowiedzialnych za biosyntezę cholesterolu jako formy obronnej, potwierdzają mechanizm działania tych związków. Najsilniejsze działanie wykazywał związek **47** ponieważ dodatkowo obniżał ekspresję genu PDHB, kodującego białko odpowiedzialne za dekarboksylację pirogronianu do Acetylo-CoA, który jest kluczowym metabolitem wielu procesów katabolicznych i anabolicznych.

Otrzymano selenosteroidy **61**, **64**, **66** i **69** w oparciu o sprzęganie terminalnych alkinów steroidowych z PhSeCl katalizowane solami miedzi(I). Została dokonana optymalizacja warunków reakcji poprzez wybór odpowiedniego rozpuszczalnika, katalizatora oraz temperatury.

Selenosteroidy **61**, **64**, **66** i **69** zostały kompleksowo przebadane pod kątem właściwości przeciwnowotworych. Zostały przeprowadzone badania oceniające stopień

hemolizy. Uzyskana wartość na poziomie 1-2,5% potwierdza ich dobrą hemokompatybilność. Wykonano również badania oceny ich kardiotoksyczności, ponieważ jednym ze skutków ubocznych terapii nowotworowej raka piersi jest niewydolność serca. Uzyskane dane potwierdzają znikomą toksyczność na komórkach H9C2. Dodatkowo oceniono cytotoksyczność uzyskanych selenopochodnych. Związki **65**, **66** i **69** w stężeniu 10 μg·mL− 1 hamowały żywotność komórek o połowę.

7. Dorobek naukowy

Publikacje

1. I. Jastrzebska, S. Mellea, V. Salerno, **P. A. Grześ**, L. Siergiejczyk, K. Niemirowicz-Laskowska, R. Bucki, B. Monti, C. Santi. „PhSeZnCl in the Synthesis of Steroidal β-Hydroxy-Phenylselenides Having Antibacterial Activity", *Int. J. Mol. Sci., 20*(9), 21212019 (**2019**)
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2. **P. A. Grześ**, B. Monti, N. Wawrusiewicz-Kurylonek, L. Bagnoli, L. Sancineto, I. Jastrzebska, C. Santi, Simple Zn-Mediated Seleno- and Thio-Functionalization of Steroids at C-1 Position, . *Int. J. Mol. Sci*. **2022**, 23, 3022.
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3. **P A. Grzes**, A. Sawicka, K. Niemirowicz-Laskowska, P. Wielgat, D. Sawicka, H. Car, I. Jastrzebska, „Metal-promoted synthesis of steroidal ethynyl selenides having anticancer activity", *The Journal of Steroid Biochemistry and Molecular Biology*, **2023**, 227, 106232.
Impact factor 5.011 Punkty ministerialne 100

4. F Mangiavacchi, I, F Coelho Dias, I. Lorenzo, **P. Grzes** ,M. Palomba, O. Rosati, L. Bagnoli, F. Marini, C. Santi, E. J. Lenardao and L. Sancineto „Sweet Selenium: Synthesis and Properties of Selenium-Containing Sugars and Derivatives", *Pharmaceuticals* **2020,** 13, 211; doi:10.3390/ph1309021
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5. I. Jastrzebska,, **P A. Grzes**, K. Niemirowicz-Laskowska, H Car, „Selenosteroids - promising hybrid compounds with pleiotropic biological activity: synthesis and biological aspects" *Journal of Steroid Biochemistry and Molecular Biology* 213 **(2021**) 105975 https://doi.org/10.1016/j.jsbmb.2021.105975
Impact factor 5.011 Punkty ministerialne 100

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Uzyskane finansowania projektu badawczego pt „Synteza i aktywność biologiczna steroidowych analogów ebselenu" w ramach Badań Młodych Naukowców w 2019 na Uniwersytecie w Białymstoku

Staże

Staż na Universita degli studi di Perugia w grupie profesora Santiego w okresie 09.01-09.05. 2019

Konferencje

1. XLVIII Ogólnopolska Szkoła Chemii „ Pod Strzechą Chemii" 28.04-02.05 2018 Karczowiska Paweł Grześ pt *„ Mój lager cuchnie… Czyli o starzeniu i leżakowaniu piwa* **komunikat**

2. *On reactions of different steroids deriatives with Santi's reagent (PhSeZnCl). The synthesis ofselenosteroids.* 24 th Conference of Isopreonoids, Białystok Poland, September 9-12 2018 **poster**

3. XXI International Symposium „Advances In The Chemistry of Heteroorganic Compounds" Łódź 23.11.2018, Izabella Jastrzębska, Paweł A. Grześ, "*Study The Reactivity of Santi's Reagent- The Synthesis Of Selenosteroids"* **poster**

4. 8rd Scientific Workshop of the multidisciplinary group SeS Redox and Catalysis (WSeS-8*)* May 30th – June 1st 2019 Perugia Italy Paweł Grześ, Izabella Jastrzębska,

Claudio Santi, Bonifacio Monti *"Simple Zn-mediated Se-Michael addition. Synthesis of steroidal Selenides"* **poster+ flash talk**

5. VI Konferencja Związki biologicznie czynne: synteza, struktura synteza 27-29.06 2019 Paweł Grześ, Izabella Jastrzębska, Claudio Santi, Bonifacio Monti „ *Using Se-Michael as an easy way for selenosteroids synthesis* **poster**

6. XXII International Symposium „Advances In The Chemistry of Heteroorganic Compounds" Łódź 22.11.2019 *"Using Zn-mediated Se-Michael addition. Synthesis of selenosteroids*" Paweł A. Grześ, Claudio Santi, Bonifacio Monti, Izabella Jastrzębska **poster**

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Review

Selenosteroids - promising hybrid compounds with pleiotropic biological activity: synthesis and biological aspects

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ABSTRACT

It is established that steroid based agents are an example of compounds obtained from natural patterns and are of great importance due to their application in the prevention and treatment of diseases. Selenosteroids are hybrids formed by attaching Se-moiety to a steroid molecule. In these types of hybrids, selenium can be present as selenide or as a part of selenosemicarbazones, isoselenocyanates, selenourea, *etc.* Attaching a Se-moiety to a biologically active steroid might enhance the biological properties of both fragments. Available literature indicates that these kinds of hybrids demonstrate significant anticancer activity, which renders them interesting in terms of medical use. In this review, we present various methods of synthesis and demonstrate that seleno-steroid compounds are promising molecules for further pharmaceutical application.

1. Introduction

The last few decades have witnessed a surge of interest in the synthesis of organoselenium-based compounds due to their pleiotropic properties and association with their promising biological activity [1–4]. Selenium, a crucial bioelement, is found in mammalian organisms in the form of selenoenzymes and selenoproteins, which play a key role as a redox modulator in protecting the cell against oxidative damage [5–7]. Based on the published literature, there are more than 50 identified selenoprotein families, despite, their role not being fully researched. For example, deiodinases are involved in the regulation of thyroid hormone activity through reductive deiodination. On the other hand, it is presumed that selenoprotein N plays a putative role during muscle development [8]. Numerous studies have concentrated on the new selena-compounds to indicate their therapeutic and protective potential. For this purpose, different types of selena-compounds based on steroid moiety have been designed and synthesized in recent years. While selenosteroids are not compounds found in nature, many of them display interesting biological properties and have been identified as glutathione peroxidase mimics, antioxidants, antineoplastic, antimicrobial, *etc.* [9]. Additionally, it should be emphasized that they are involved in other biological processes which might exert an impact on their pharmaceutical applications. In effect, evaluation of their mimetic, such as selenosteroids might provide additional information about their function. Moreover, it could also lead to the development of new targets for the treatment of different diseases, including cancerand those that possess metabolic etiology. However, in most cases, a lack of complex biological studies is observed, and in effect, their characteristics are not fully known while their potential remains hidden. Selenosteroids (SeSt) are compounds formed by attaching a selenium-moiety to a steroid system (Fig. 1). According to their structure, selenosteroids can be divided into two groups: In the first group, selenium is directly connected to the steroid molecule to form alkyl or aryl selenide (Fig. 1a-b). In the second group, selenium is introduced to compounds in the form of selenourea, benzisoselenazolones, *etc.* (Fig. 1c-d).

The review is divided into two parts. In the first part, the methods of selenosteroids synthesis are presented, while in the second one their biological activity is discussed. In this review, we would like to present synthesis methods in which a Se-moiety is attached directly to a steroid molecule and in which selenium is present in a group connected with the steroid molecule. The data will not be specified chronologically, but rather non-linearly in order to reveal a narrative related to the

Abbreviations: ADD, 1,4-androstadiene-3,17-dione; AIBN, azobisisobutyronit; BHT, butylated hydroxytoluene; BSA, benzeneneseleninic anhydride; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GPx, glutathione peroxidase; MCPBA, *m*-chloroperoxybenzoic acid; ROS, reactive oxygen species; SeSt, selenosteroid; TBTMG, *t*-butyl-*N'*, *N'*,*N'*,*N''*-tetramethylguanidine; THF, tetrahydrofurane; THP, tetrahydropyran.

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procurement and usage of selenosteroids.

2. Selenosteroids synthesis

2.1. Synthesis methods in which a Se-moiety is attached directly to a steroid molecule

The free-radical addition of selenosulfonate (p-tolyl-SO_2SePh) to readily available acetylenic steroidal starting material **1** caused the production of selenosteroid **2** as 1,2-adduct with high regio- and stereoselectivity. Selenosulfonation gave 1,2-adduct with 80 % yield. The obtained Se-compound **2** was a crucial intermediate for the synthesis of marine sterols (24,28-*dehydroaplysterol* and *xestosterol*) containing a 24-methylene moiety. The synthesis pattern is presented in Scheme 1a [10]. The attainment of SeSt **2** occurred through the reaction with radicals formed *via* $PhSeSO_2Ar$ decomposition (Scheme 1b) [11].

Synthesis of biologically active steroid substitutes *via* selenosteroid **4** was described [12]. The literature revealed that the elimination of selenoxide resulted in olefin production [13]. This methodology, known as the selenenylation-elimination procedure, was used in the synthesis of 15β-hydroxysteroids, which are steroids of the human perinatal period. An example of the application of selenoxide elimination in the synthesis of 15β,17α-dihydroxypregnenolone (**6**) is presented in Scheme 2.

Pavoninin-1 is a shark repellent substance. One of the final steps in the preparation of *pavoninin-1* was the reaction to obtain the α,β-unsaturated ketone steroidal aglycone. For this purpose, selenenylation of steroidal ketone **7**, oxidation of the resulting selenide **8**, followed by elimination were used [14]. The reaction sequence is shown in Scheme 3.

The biological activity of some D-ring-secoestrogens led to the production of different secosteroids related to dehydroepiandrosterone (DHEA). To obtain D-ring-secosteroids, oxidation of the steroidal selenide **10** to the selenoxide **11** followed by the seleno-Pummerer reaction were applied (Scheme 4). The 16-acetoxyselenide diastereomers **12** were converted into secosteroid **14** [15].

The detection and evaluation of steroid residues in biological materials are essential for pharmacological examinations. For this purpose, the synthesis of ^{13}C-labeled steroids was performed. The key step of ^{13}C3-androstanes synthesis includes a hetero-Michael reaction that is composed of 1,4-androstadiene-3,17-dione (ADD). One of the Michael adducts was selenosteroid **15**, which had undergone an ozonolysis reaction (Scheme 5). The result **16** was then transformed into *[2,3,4-^{13}C3]-ADD* [16].

Estrogens were treated with benzeneselenyl halides (PhSeCl and PhSeBr) and as a result of the estradiol (**17a**) and estrone (**17b**) reaction with benzeneselnyl chloride 2-phenylselenyl steroidal derivatives **18a** and **18b** were obtained (Scheme 6). In addition, 4-phenylselenylestrogens were observed (10 % yield for 4-phenylselenylestradiol and 12 % for 4-phenylselenylestradione). A corresponding process of PhSeBr with estradiol gave a product of steroid halogenation (2- and 4-bromo compounds). There was no observable reaction of estrogens with phenylselenyl iodide [17]. 2-Phenylselenylestrogens were then converted into the corresponding 2-halogenated estrogens.

Steroidal phenylvinylselenides **19-20** were products of ketonic hydrazones reaction with PhSeBr in the presence of *t*-butyl-*N*',*N*',*N*",*N*"-tetramethylguanidine (TBTMG) [18]. The yields obtained in this protocol were high, 87 % for **15a** and 83 % for **15b**. The reaction occurred *via* the diazonium ion (*i*), followed by N_2 abstraction. The formed phenylselenium cation (*ii*) was then attacked by a bromine anion followed by an elimination process. The presumed mechanism of the process is presented in Scheme 7.

Another example of a phenylselenyl group introduced into the steroid molecule was achieved through reaction with PhSeCl [19]. Trisubstitued olefins (steroidal 5,6-alkenes) were treated with phenylselenyl chloride in methanol. As a result of cholesterol reaction with selenylchloride, steroidal selenide **21** was achieved with 62 % yield (Scheme 8a). Extending the reaction time caused a regio- and stereoselective deselenylation process. The probable mechanism of deselenylation is presented in Scheme 8b. Presumably, the involvement of the neighbouring MeO- group in the displacement of selenonium chloride to generate the oxonium ion occurred. Subsequently, the nucleophile reacted with both bridged carbons (at C5 and C6 in the steroid molecule).

A one-pot procedure to generate F_3CSeCl was described [20]. The reagent for selenylation F_3CSeCl was readily prepared from $BnSeCF_3$ and SO_2Cl_2. One of the obtained products of direct trifluoromethylselenelation was 2-trifluoromethylselenacholestan-3-one (**22**), presented in Scheme 9. The reaction of cholestan-3-one with F_3CSeCl was highly regio- and stereoselective. Fluoroselenylation occurred in a less hindered C-2α position which is in anti-position to the 19 methyl group [21].

According to research, regio- and stereoselectivity of electrophilic addition to precisely defined cyclohexene systems present in steroidal molecules can be controlled by neighbouring groups [22]. For this study, selenosteroids **24-25**, presented in Scheme 10, were obtained. Steroidal Se-derivative **24-25** were achieved by phenylselenylation with PhSeCl in the presence of thallium(I) trifluoroacetate. Observed yields of selenylation were excellent (more than 95 %).

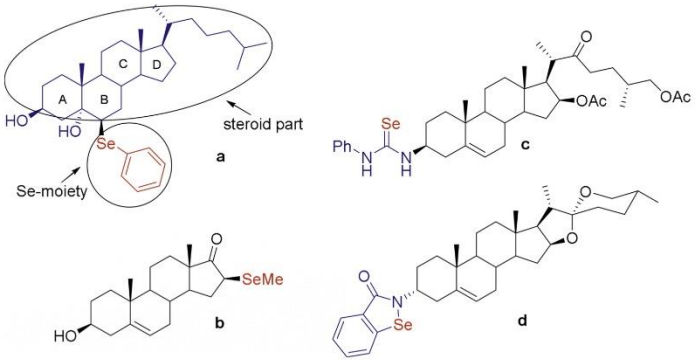
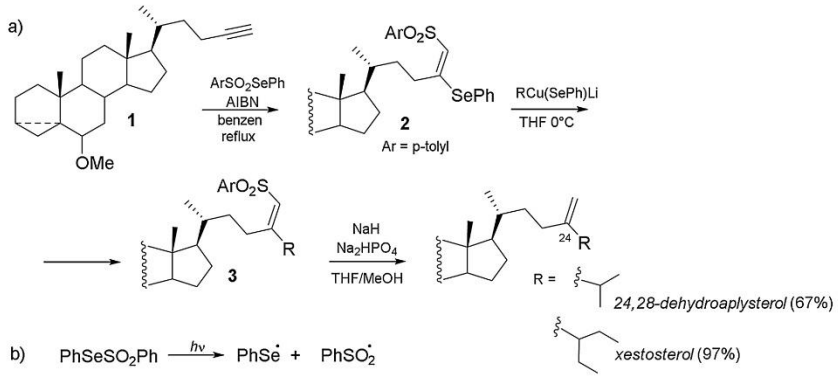
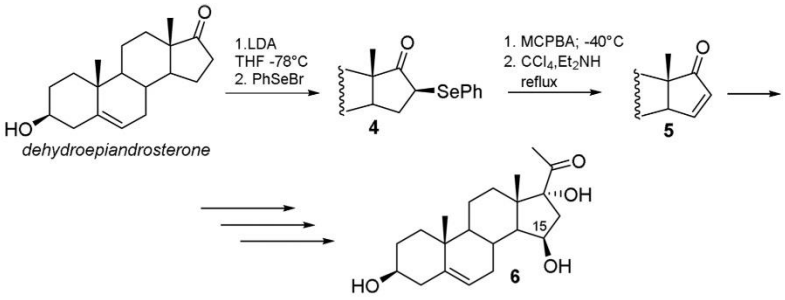


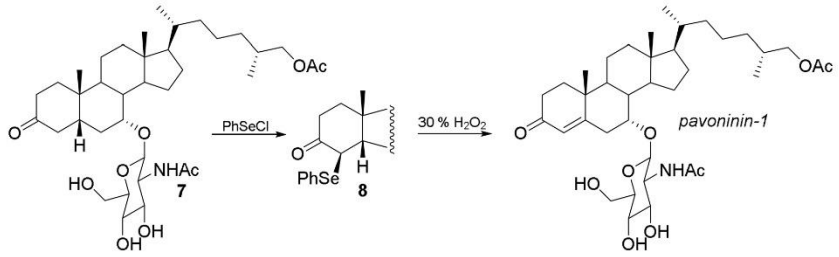
Fig. 1. Examples of selenosteroids. Selenium is bonded directly to the steroid molecule (**a** and **b**), selenium is present in a group (in blue) bonded to the steroid (**c** and **d**).



Scheme 1. Synthesis of marine sterols *via* selenosteroid **2**.



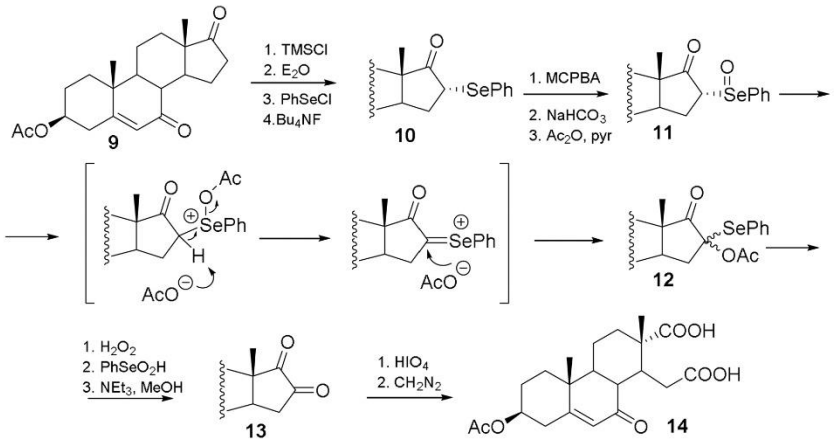
Scheme 2. Synthesis of 15β-hydroxysteroid *via* selenosteroid **4**.



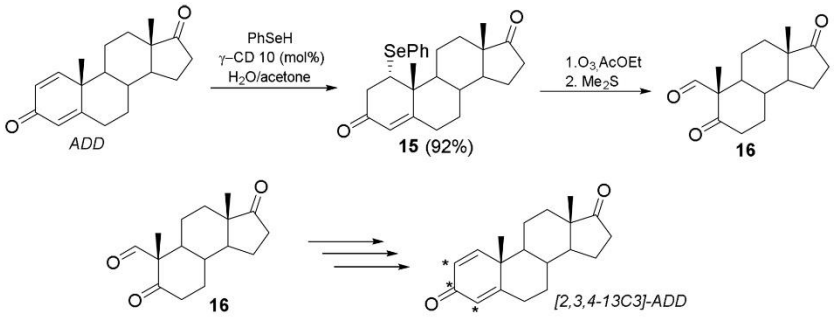
Scheme 3. The selenenylation-elimination protocol used in the synthesis of *pavoninin-1*.

The spiroketal moiety of spirostane sapogenins is sensitive to protic and Lewis acids [23]. As a result of the reaction of steroidal spirostanes with benzeneselenic anhydride (BSA) in the presence of $BF_3{\cdot}Et_2O$, a C-23 phenylselenide derivative **26** was formed [24]. The reaction of tigogenin acetate with BSA/$BF_3{\cdot}Et_2O$ afforded 23-phenylselenide **26a** with 30 % yield. An analogous reaction of sarsasapogenin acetate created a similar product **26b**. The reaction mechanism was proposed by the authors. The reactions of spirostane sapogenins with BSA proceeded *via* enol ether intermediate (*i*). Scheme 11 shows the synthesis of SeSt **26**.

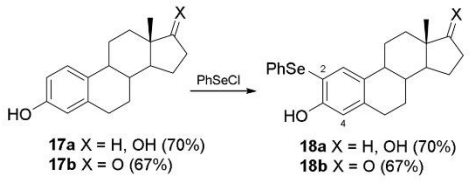
Spirostanol glycoside dioscin is a secondary metabolite that represents a broad spectrum of antitumor, antifungal, and anti-inflammatory activity [25]. To check the cytotoxic activity of *selenadioscin*, the selena analog of dioscin was obtained. Synthesis of *selenodioscin* was started from pseudodiosgenin **27** readily available from commercial diosgenin. The key step was steroidal diselenide **28** preparation followed by cyclization to 26-selenodiosgenin **30** (Scheme 12) [26].



Scheme 4. Synthesis of D-ring-seco steroid-using the seleno-Pummerer reaction.



Scheme 5. Synthesis of *[2,3,4-^{13}C3]-ADD via* selenosteroid **15**.



Scheme 6. Synthesis of 2-phenyl estrogens **18a** and **18b**.

2.1.1. Methods synthesis of selenosteroids as potential scanning agents for hormone receptors

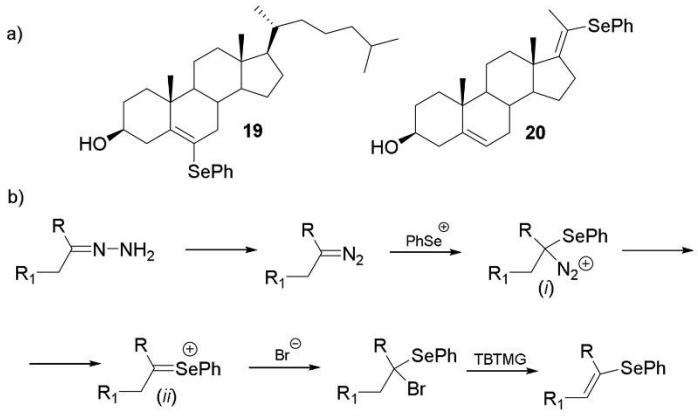
Selenoestrogens were obtained for studies of noninvasive discrimination between hormone-dependent and hormone-independent mammary tumours [27]. ^{75}Selenium-labeled compounds were selected based on satisfactory scanning qualities, the long half-life, ease of chemical procedures, and significant biological stability. To obtain 7α-(phenylseleno)estra-trienol **32**, the electrophilic addition of arylselen bromide and acetate was applied to olefin **31** [28]. The reaction of Δ^6-estardiol diacetate is depicted in Scheme 13.

Other potential estrogen-receptor scanning agents were prepared using a different approach (Scheme 14) [29]. Se-labeled steroidal estrogen **36a** was obtained from 16,17-olefin **34**, *via* epoxidation with m-chloroperoxybenzoic acid (MCPBA) followed by selenenylation with dimethylaluminium methylselenolate [30]. (Phenylseleno)estrone **37** was a product of a lithium enolate **35** reaction with PhSeCl.

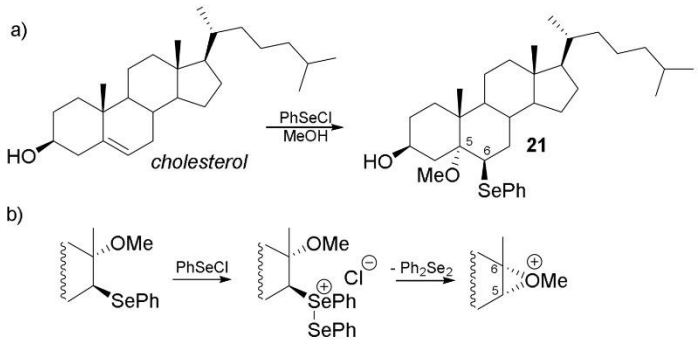
Synthesis and binding affinity studies of Se-substituted estrogens were described [31]. The crucial step of 20-lithio derivative **39** generation and subsequent introduction of selenium moiety was achieved by using dimethyl diselenide or diphenylselenide. The methylseleno analogues had a higher affinity for estrogen receptors than the corresponding phenylseleno analogues. The SeSt **40** synthesis is presented in Scheme 15.

Selenosteroids obtained by the methodology shown in Schemes 14 and 15 were tested for estrogenic and antiestrogenic activity [32]. It was proven that Se-analog of ethynylestradiol **40** and its SePh-derivative showed the highest levels of estrogenic activity.

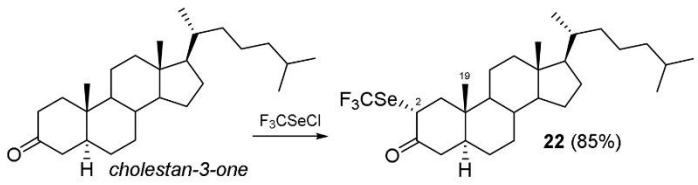
For further study of binding affinity assay, 17α-E- and 17α-Z-phenylselenovinyl estradiols (**41a** and **41b**, respectively) were obtained [33]. For this purpose, electrophilic destannylation was applied (Scheme 16). The process required the use of PhSeCl followed by hydrolysis. Compounds **41a** and **41b** were achieved with 85 % and 90 %



Scheme 7. a) Structures of steroidal phenylvinylselenides **19-20**; b) Possible mechanism of the reaction.



Scheme 8. a) The reaction of cholesterol with PhSeCl; b) The probable mechanism of deselenylation.



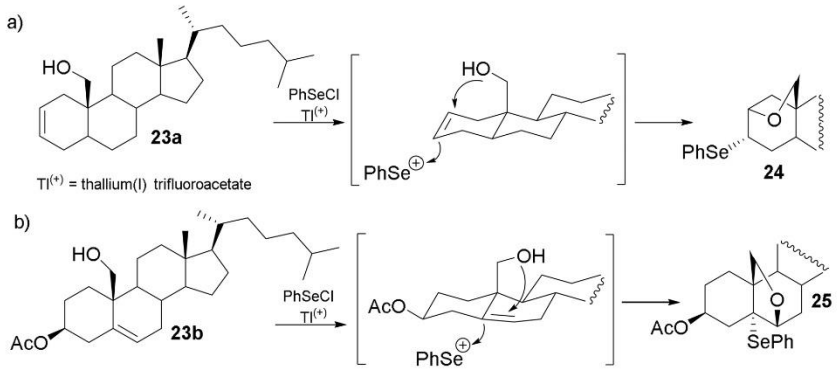
Scheme 9. The trifluoromethylselenylation of cholestan-3-one.

yield, respectively. 17α-E- and 17α-Z-phenylselenovinyl estradiols **41a-b** were characterized by higher binding affinity than methylseleno estradiol **40** and its phenylseleno derivative.

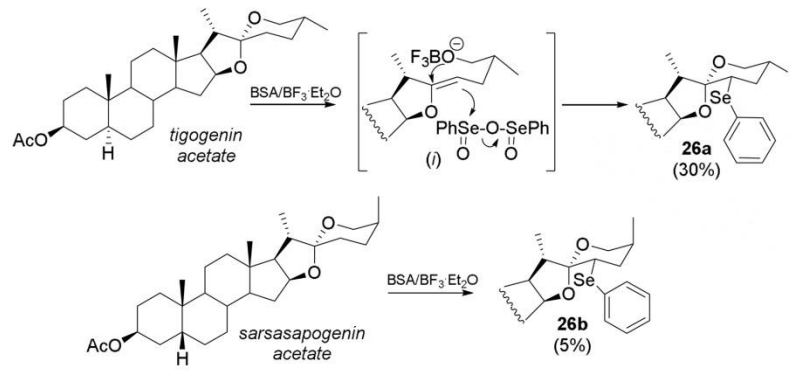
Steroid hormones (progesterone and testosterone) containing organoselenium substituents were prepared with one simple reaction. In order to do so, 3,3-(ethylenedioxy)pregnenon (**42**) was treated with nBuLi and PhSeCl followed by hydrolysis. 21-(Phenylselenyl)progesterone **44** was obtained, as shown in Scheme 17. Progesterone Se-derivatives **44-45** and testosterone derivatives **46a-b** were tested for binding affinity to different steroid hormone-receptors. It was proved that only SeSt **44** was effective for binding to the progestin-receptor [34].

2.1.2. Selenosteroids obtained as a product of epoxide opening reaction

Nucleophilic opening of epoxides leads to 1,2-functionalized systems. Commonly, such cleavages occur stereospecifically to trans isomers. The steroidal epoxide was opened using selenol in the presence of neutral chromatographic alumina (activity "Super I" on the Brockmann scale). The heterogeneous alumina-promoted reaction of epoxy cholestane **47** with PhSeH generated regio- and stereospecifically *trans*



Scheme 10. The reaction of cyclohexene **23a-23b** with PhSeCl/ thallium(I) trifluoroacetate.



Scheme 11. The reaction of tigogenin acetate and sarsasapogenin acetate with BSA/BF_3·Et_2O.

hydroxyselenide **48** with 72 % yield [35]. The result is presented in Scheme 18.

Two protocols were implemented to introduce selenium into the steroid system from steroidal epoxide **49** [22]. In the first method, the epoxide was treated with diselenide followed by the reductive cleavage of the Se-Se bond using $NaBH_4$. The second protocol used selenolate species, which were obtained by reductive cleavage of the Se–Se bond of diselenide using Zn and HCl (ultrasound was used). Superior yields were obtained with Zn/HCl/ultrasound protocol (90 % *vs* 63 %). *Trans*-hydroxy steroidal selenides **50** were obtained as a result of the SN_2 mechanism [36] (Scheme 19).

Reactions of epoxysteroids with *in situ* formed PhSeZnCl were performed [37]. PhSeZnCl (Santi's reagent), readily prepared through the oxidative insertion of elemental zinc into Se-Cl bond of phenylselenyl chloride, is a modern nucleophilic selenium reagent [38]. The experiment demonstrated that PhSeZnCl reacted exclusively with 5α, 6α-epoxycholestane **51a** to give steroidal β-hydroxy-phenylselenide **52**, as presented in Scheme 20.

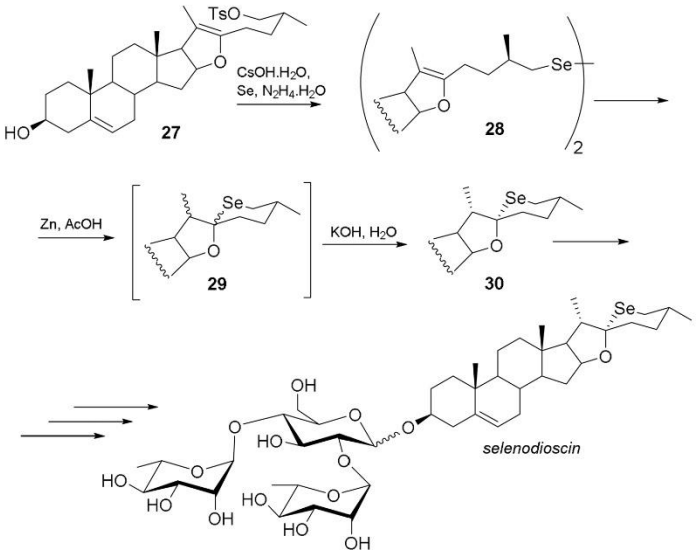
As a result of 5α,6α-epoxysteroid reaction with sugar diselenide in the presence of a reducing agent ($NaBH_4$), steroidal Se-glycosides were observed [39]. Regio- and stereoselective epoxide opening resulted in the formation of steroidal derivatives in *trans*-diaxial positions. As an example of the steroidal Se-glycoconjugate synthesis, Scheme 21 shows the synthesis of SeSt **57** from 5,6-epoxystigmasterol **56**. Many other examples of selenium-containing sugars have been described [40].

2.2. Synthesis methods in which selenium is present in a group connected with the steroid molecule

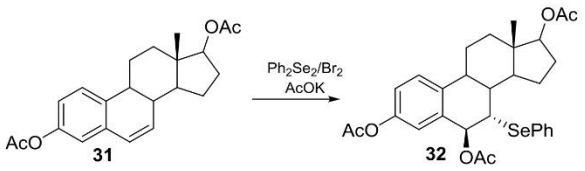
Encouraged by the antioxidant activity of selenium compounds that mimic glutathione peroxidase (GPx), it was an inviting idea to link a selenium atom with a steroid molecule [41,42]. The attachment of a selenium moiety to a biologically active steroid created a compound that may enhance the biological properties of both fragments. In this section, synthesis of SeSt with antiproliferative or/and antioxidant activity is presented.

In order to check GPx activity, diosgenin-based selenourea **61** synthesis was accomplished [43]. The key process in the protocol was the reaction of a steroidal amine **59** with phenyl isoselenocyanate **60** (Scheme 22). Tests revealed that obtained selenourea **61** behaved as an excellent GPx mimic and possessed promising antiproliferative activity.

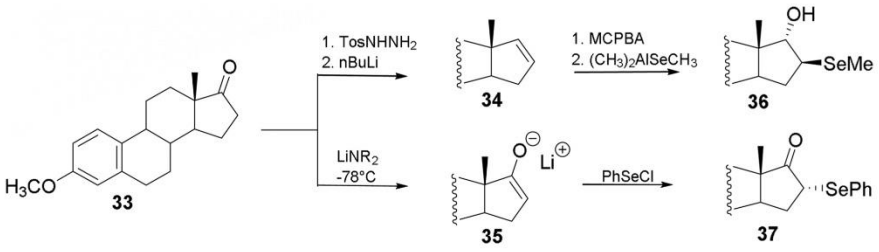
Synthesis of several Se-derivatives started from diosgenin, hecogenin, smilagenin, and estrone [44]. Regarding antiproliferative activity, different organoselenium moieties (benzeneselenazoles, selenosemicarbazones, isoselenocyanates, selenoureas, and diselenides) were introduced into steroid molecules for structure-relationship activity



Scheme 12. Synthesis of *selenodioscin*.



Scheme 13. Synthesis of 7α-(phenylseleno)estra-trienol **32**.



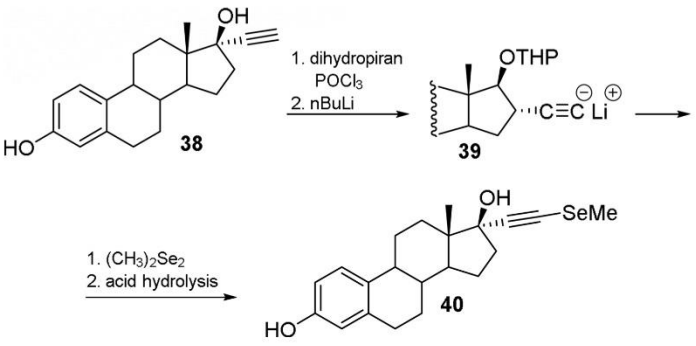
Scheme 14. Synthesis of selenosteroids **36-37** [29].

study (Fig. 2).

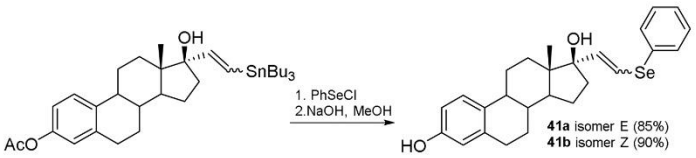
Additionally, the new SeSt were tested for antioxidant activity [44]. Significantly, some of the described steroidal Se-derivatives were excellent GPx mimics. Scheme 23 presents the convergent synthesis of steroidal ebselen analogues **62-64**.

Synthesis of subsequent benzisoselenazolones (ebselen analogues) steroidal Se-derivatives **67-68** was performed through the process of oxime **65** reduction to a steroidal amine **66** [45]. The obtained amine **66** was then subjected to a reaction with previously synthesized, appropriately substituted selenium chloride **67** (Scheme 24). Antitumor activity of obtained steroidal ebselen analogues **68** and **69** was tested. The results of the biological assay showed promising activity in the case of selected synthesized Se-compounds.

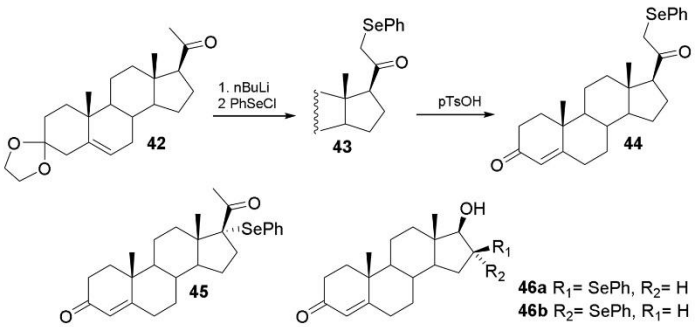
Steroidal selenazoles **72** were prepared for evaluation of their antiproliferative activities against several types of human carcinoma [46]. Pregneneolone was used as a starting material. The reaction of



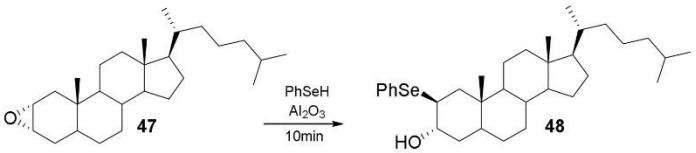
Scheme 15. Synthesis of 17α[(methylseleno)ethynyl]-17β-estradiol (**40**).



Scheme 16. Electrophilic destannylation of estradiol derivatives.



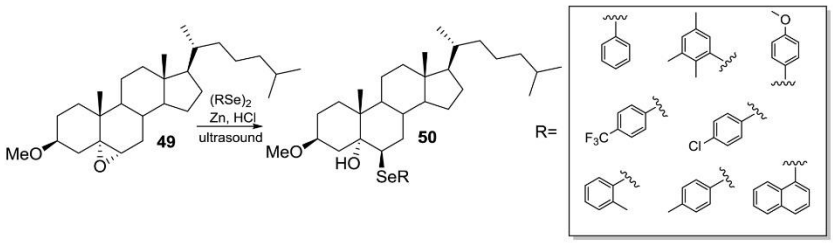
Scheme 17. a) Synthesis of phenylselenium-substituted steroid hormone **44**; b) Structure of selenosteroids **45-46**.



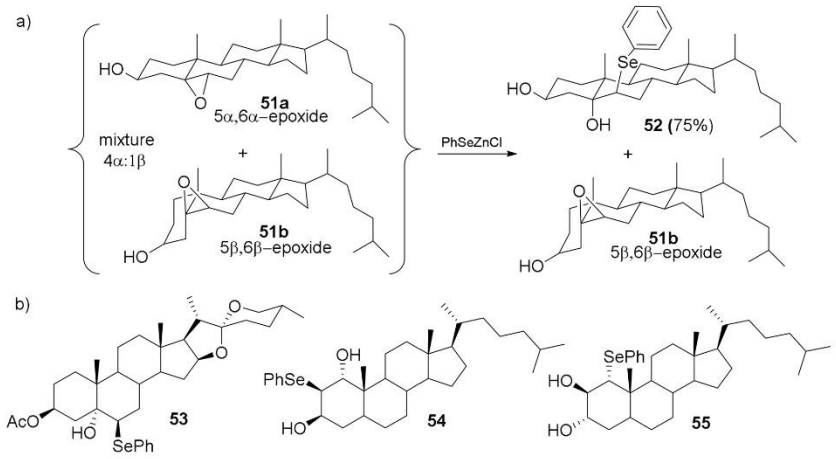
Scheme 18. The steroidal epoxide opening at alumina surface.

3-substituted steroid **70** with semicarbazide gave the corresponding semicarbazone **71,** which was then transformed into pregnenolone-17-[1′,2′,3′] selenodiazole **72** (Scheme 25). All information on the obtained results of *in vitro* antiproliferative activities is summarized in Table 1.

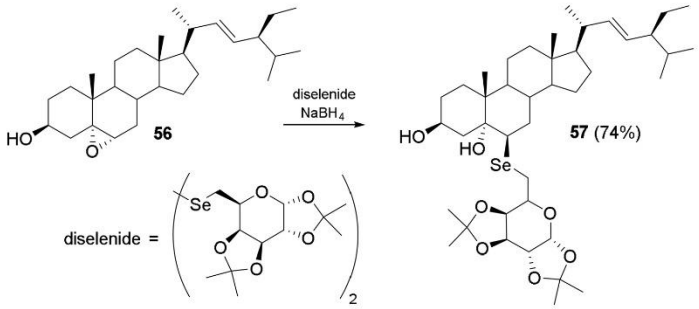
Several steroidal (phenylseleno)formates derivatives **75** were obtained as precursors of carbon-centered radicals [47]. The procedure of Se-derivative **75** formation was as follows: steroidal alcohol **73** was reacted with 20 % solution of phosgene to afford steroidal chloroformate **74**, which was then reacted with phenylselenoate. The aforementioned



Scheme 19. Synthesis of selenosteroids **50**.



Scheme 20. a) The reaction of 3β-hydroxy-5α,6α-epoxycholestane (**51a**) with *in situ* generated PhSeZnCl; b) structures of other obtained SeSt **53-55** using PhSeZnCl.

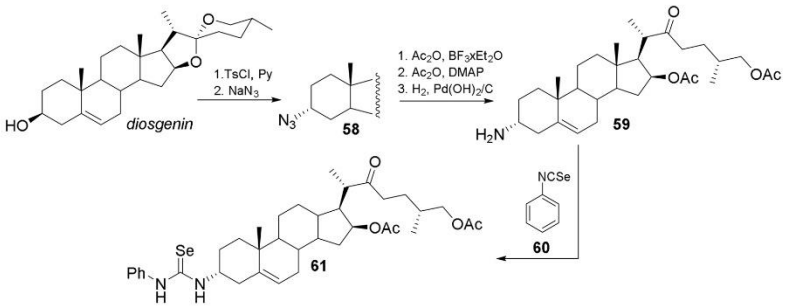


Scheme 21. Synthesis of compound **57**.

procedure is outlined in Scheme 26.

3. Selenosteroids biological activity

In this part, the focus is on pleiotropic potential selenosteroids. Based on a recently published study, their potential as antioxidant and antiproliferative agents to restrict tumor growth as well as their ability to reduce the metabolic activities of bacteria cells and protective role in the formation of biofilm are discussed. It should be emphasized that identification of compounds that possess antineoplastic activity in an era of increasing cancer incidence as well as/or antimicrobial efficacy in addition to an increase in drug-resistance phenomena are a great



Scheme 22. The synthesis of diosgenin-based selenourea **61**.

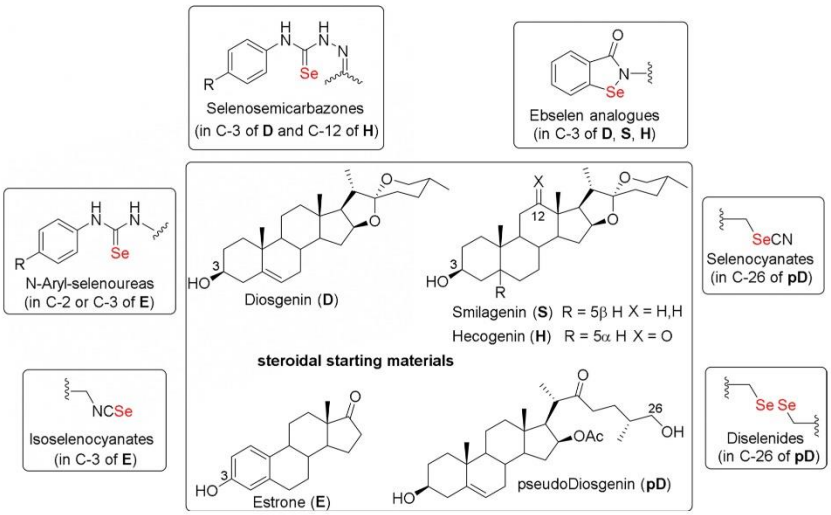


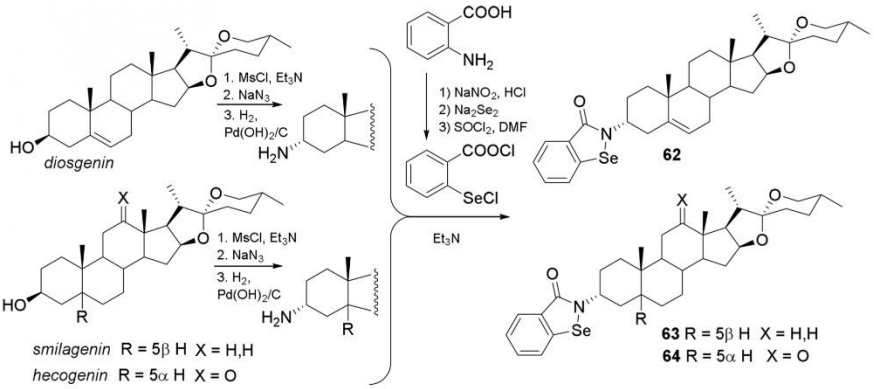
Fig. 2. The seleno-derivatives design [44].

challenge of modern medicine and pose an actual problem in health-care systems.

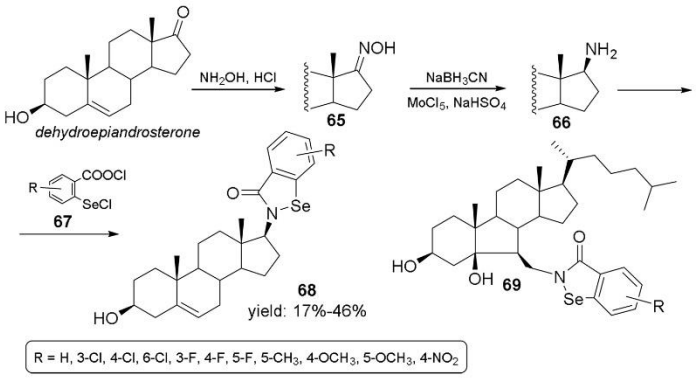
The development of civilization and our frequently unhealthy lifestyle resulting from it has led to an increase in cancer incidence [48]. In effect, it has been established that cancer is one of the leading causes of unnatural death in the world [49]. It is characterized by the presence of abnormal cells that divide in an uncontrolled manner and invade nearby tissues. Additionally, cancerous cells might spread to other parts of the body *via* the blood and lymph systems. Of the various types of cancer, the most common organs affected by the neoplastic process are the lungs, the breasts, colon, and the stomach [50]. Besides recent progress in medicine, treatment of malignancy that involves chemo – and radiotherapy, as well as surgery, are associated with high toxicity, development of chemo resistance and occurrence of undesirable and traumatic side effects [51]. In light of the above, the search for a new strategy involving the creation of novel families of chemotherapeutic agents is a pivotal task in the pharmaceutical industry.

Recent decades have indicated that natural products have gained great attention due to their application in cancer prevention and treatment [52,53]. It should be emphasized that over 60 % of approved anticancer drugs, such as taxol, vinblastine and camptothecin, as well as molecules that possess promising anticancer activity, such as steroidal analogues, *e.g.* diosgenin and its derivatives are derived from a natural source [54]. In light of this, steroids are attractive polycyclic biomolecules that possess a wide range of activities [55]. Due to their high lipophilic nature, they might easily enter into most kinds of cells *via* passing the cell membrane by simple diffusion as well as through interaction with intracellular receptors [56]. This means that they are interesting components/basis of hybrid molecules and play the role of vehicles for targeting different pathologies [57]. It is established that the structure of compounds determines their biological properties. In effect, the creation of novel steroid derivatives including modification in structural features of the steroid moiety, presence of heteroatoms, and their side chains provides a way to modulate their function. Therefore, obtaining new curative agents for the treatment of crucial pathologies, such as cancers and infectious diseases, is a significant opportunity.

Thus, in order to overcome the problems associated with ineffective cancer treatment and to achieve higher compatibility against host cells,



Scheme 23. The synthesis of steroidal ebselen derivatives **62-64**.



Scheme 24. Synthesis of dehydroepiandrosterone seleno derivative **68** and structure of SeSt **69**.

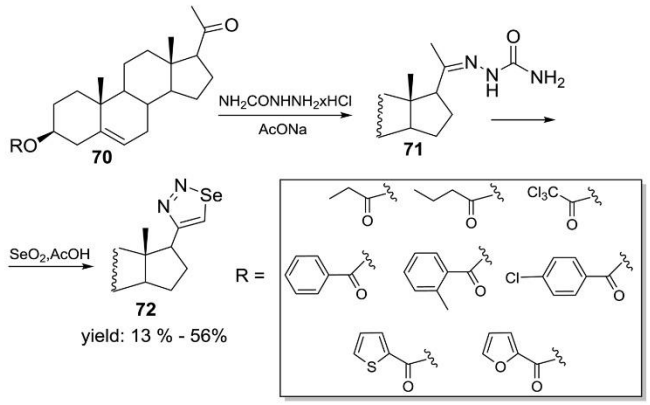
several studies including introducing new molecular elements such as heteroatoms, functional groups, or use of drug carriers have been described. Due to this in this work we focus on the potential application of organoselenium derivatives of steroid compounds. To date it has been established that this class of compound exerts relevant biological activities such as anticancer, anti-inflammatory and antimicrobial properties. Significantly, their strong antioxidant activities, which provide the possibility of applying them to eliminate different form of oxygen- and nitrogen- reactive species among others, are attractive agents in terms of their application in cancer prevention and treatment.

The aforementioned features mean that organoselenium steroid derivatives are currently considered as promising agents with pleiotropic activities that demonstrate cytotoxicity against malignant cells at lower doses than normal cells (Fig. 3).

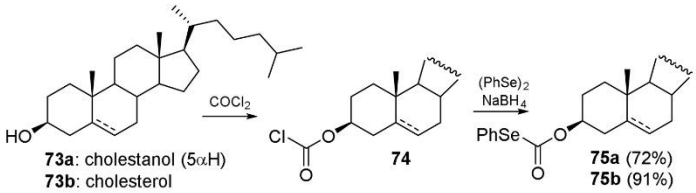
Nevertheless, the number of papers focusing on selenosteroids is extremely scarce, while the presented studies are incomplete and can only give partial data that do not offer enough grounds to draw particular conclusions. The data relating to the biological activity of synthesized derivatives of steroids with organoselenium moiety are summarized in Table 1.

IC50 - half inhibitory concentration; GI50- the concentration of the compound required for reducing the cancer cell proliferation rate to 50 % of its normal value. Cell line: HeLa -human cervical cancer; MDA-MB-231 - estrogen negative breast cancer; MCF-7 – estrogen-positive breast cancer; OVCAR-3 – human ovarian adenocarcinoma; HepG2 - liver hepatocellular carcinoma; NIH3T3 - mouse fibroblasts; A-549 - non-small lung cancer; K-526 - chronic myelogenous leukaemia; L-02 - normal human liver; HBL-100 - human milk-derived transforming breast cancer; T-47D - human breast cancer; WiDr – human colon adenocarcinoma; SKOV3 - human ovarian carcinoma.

It has been proven that compounds with antioxidant properties might protect cells from disruption of oxidative balance *via* neutralizing the generation of reactive oxygen species (ROS) [58]. It is of great significance as oxidative damage plays a crucial role in the etiopathology of various diseases, including cancer [59]. ROS caused oxidative damage to basic cell components, such as lipids, protein and DNA, and then play a role in the initiation of cancer angiogenesis, metastasis, and survival of neoplastic cells [60,61]. Increased ROS levels, associated with down-regulation of cellular antioxidant enzyme systems, caused malignant transformation *via* different molecular targets and pathways, such as NF-κB. Recently published reports demonstrate that the application of natural products rich in antioxidant compounds might result in



Scheme 25. The synthesis of pregnenolone-17-[1′,2′,3′]selenodiazoles **72**.



Scheme 26. The synthesis of steroidal (phenylseleno)formates derivatives **75a-b**.

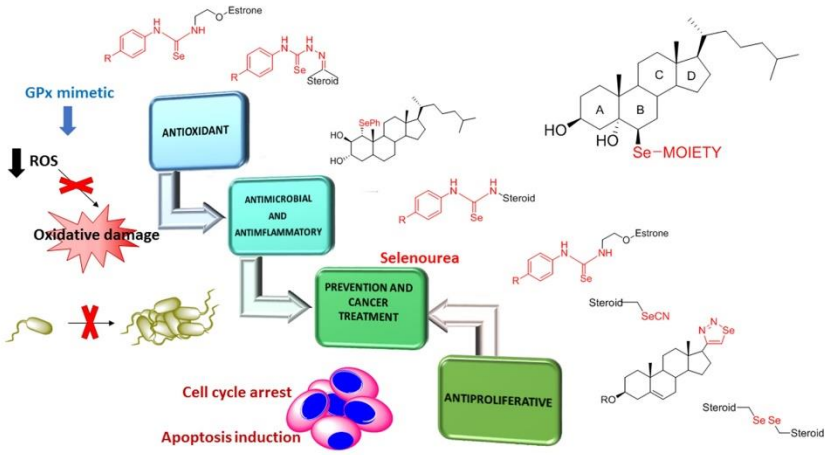
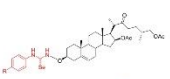
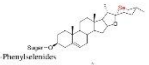
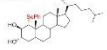
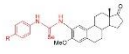
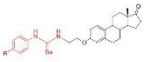


Fig. 3. Pleiotropic activities of selenosteroids.

a reduction in the incidence of several diseases, such as cancer [54,58]. The aforementioned activity is connected to the fact that, in the case of agents derived from a natural source, antioxidant properties are well correlated with antiproliferative activity. In effect, it is important to seek natural antioxidants with anticancer potential.

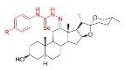
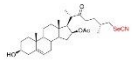
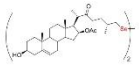
Romero-Hernández et al. indicated that among tested derivatives, *N*-phenyl selenourea showed the greatest biological activity - including excellent free-radical scavenger and a GPx mimetic [43]. Notably, this

Table 1
Studies related to the biological properties of steroids with organoselenium moiety.

Entry	The compound		Cell line	IC50/GI50 [nM]	Antioxidative	Antiproliferative	Antimicrobial	Antiinflammatory	Lit
1.	Selenourea 		HeLa MDA MB 231 MCF-7 OVCAR-3 HepG2 NIH3T3 A 549 K562	1.87 ± 0.47 6.39 ± 1.40 3.17 ± 2.08 14.59 ± 3.45 6.48 ± 0.75 7.35 + 1.92 5.00 ± 0.16 4.96 + 0.15	–	+	N/A	N/A	[43]
2.	Selenodiosgin 		L-02	7.35 ± 0.14	N/A	+	N/A	N/A	[26]
3.	Steroidal β Hydroxy-Phenylselenides 		N/A	N/A	–	+	–	N/A	[37]
4.	Selenourea 	R = H	A 549 HBL 100 T 47D HeLa WiDr A 549 HBL-100	2.2 ± 0.2 2.5 ± 0.3 3.9 + 0.6 2.1 ± 0.4 3.6 ± 0.4 2.4 + 0.4 2.4 ± 0.1	–	+	N/A	N/A	
		R = Me	T-47D HeLa WiDr A-549 HBL-100	3.0 ± 0.8 2.1 ± 0.3 2.8 ± 0.7 2.7 ± 0.3 2.3 ± 0.4	–	+	N/A	N/A	
		R = OMe	T-47D HeLa WiDr A-549 HBL-100	3.2 ± 1.0 2.3 ± 0.5 3.3 ± 1.0 2.5 ± 0.2 2.3 ± 0.2	–	+	N/A	N/A	[44]
		R = Cl	T-47D HeLa WiDr A 549 HBL 100	3.5 ± 0.7 2.0 ± 0.4 3.4 ± 0.6 2.0 ± 0.4 2.2 + 0.1	–	+	N/A	N/A	
		R = Br	T-47D HeLa WiDr A 549 HBL 100	2.8 ± 0.5 2.0 ± 0.5 3.3 + 1.7 6.0 ± 1.1 >100	–	+	N/A	N/A	
		R = Me	T 47D HeLa WiDr A 549 HBL 100	7.9 + 1.4 61 – 16 7.3 + 0.2 4.4 + 1.4 50.0 ± 6.8	–	+/–	N/A	N/A	
5.	p-Tolylselenoureido-ethoxyestrone analogues 	R = Cl	T 47D HeLa WiDr	76 – 23 31.0 + 6.1 64 ± 15	–	+/–	N/A	N/A	[44]
6.		R = Me	A 549 HBL 100	3.7 + 0.9 3.8 + 1.0	–	+	N/A	N/A	[44]

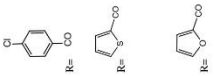
(continued on next page)

Table 1 (*continued*)

Entry	The compound		Cell line	IC50/GI50 [nM]	Antioxidative	Antiproliferative	Antimicrobial	Antiinflammatory	Lit
				13.0 + 1.7					
	Selenosemicarbazones 		HeLa WiDr A 549 HBL-100	2.9 + 0.5 8.1 ± 0.3 4.6 + 1.1 33 – 14					
		R = OMe	T-47D HeLa WiDr A-549 HBL-100	58 – 28 4.5 + 1.6 31 – 15 1.7 ± 0.5 2.2 ± 0.2	N/A	–	N/A	N/A	
7.	Selenocyanate 		T-47D HeLa WiDr A-549 HBL-100	3.2 ± 0.8 3.3 ± 0.4 5.5 + 1.4 N/A N/A	N/A	+	N/A	N/A	[44]
8.	Dimeric diselenide 		T-47D HeLa WiDr	N/A N/A N/A	–	N/A	N/A	N/A	[44]
		R = H	SKOV3 PC-3 T47D MCF-7 HEK 293 T	57.59 ± 0.86 37.35 ± 0.06 71. 28 – 1.31 >100 >100					
		R=	SKOV3 PC-3 T 47D MCF 7 HFK 293 T	42.35 + 0.66 13.24 ± 0.41 40.65 ± 0.10 >100 >100					
		R=	SKOV3 PC 3 T 47D MCF 7 HFK 293 T	36.60 + 1.05 31.74 + 0.32 41.65 + 0.24 >100 >100					
9.	Selenoandrostylpregnenolone 	R=	SKOV3 PC-3 T 47D MCF-7 HEK-293 T SKOV3 PC-3	55.31 ± 11.02 56.31 ± 2.01 >100 >100 >100 35.79 + 0.47 12.48 ± 0.44	N/A	N/A	N/A	N/A	[46]
		R= 	T-47D MCF-7 HEK-293 T SKOV3	>100 >100 >100 >100					
		R=	PC-3 T-47D MCF-7 HEK-293 T SKOV3	>100 >100 >100 >100 >100					
		R=	PC-3 T 47D MCF 7	59.85 ± 5.48 >100 >100					

(continued on next page)

Table 1 (*continued*)

Entry	The compound	Cell		Antioxidative	Antiproliferative	Antimicrobial	Antiinflammatory	Lit
		Cell line	IC50/GI50 [nM]					
	 Re=	HEK-293 T	>100					
		SKOV3	>100					
		PC-3	>100					
		T-47D	>100					
		MCF-7	>100					
		HEK-293 T	>100					
		SKOV3	33.88 ± 0.13					
		PC-3	18.56 ± 0.82					
		T-47D	37.39 ± 0.60					
		MCF-7	>100					
		HEK-293 T	>100					
		SKOV3	18.26 ± 0.49					
		PC-3	21.40 ± 0.37					
		T-47D	46.67 ± 0.19					
		MCF-7	>100					
		HEK-293 T	>100					
		A-549	25 ± 9					
		HBL-100	13 ± 3					
10.	Ebselen	T-47D	90 ± 15	+	+	+	+	[62,63]
		HeLa	28 ± 4					
		WiDr	>100					

compound showed strong antiproliferative properties as well as the ability to induce apoptosis against tested human tumour cells, with higher potency than diosgenin which has been mostly used as a starting material for the synthesis of steroidal drugs [62,63]. Moreover, this selenourea has also been proven to efficiently eliminate the ROS produced endogenously by HeLa cells. In another report, Chen et al. presented the synthesis and evaluation of cytotoxicity of two anomers of selenodioscin, derivatives of a representative of spirostan saponin – dioscin [26]. Their results indicated that β- configurations of glycosides play a crucial role in the cytotoxicity of the tested agent, while substitution of oxygen at position 26 in dioscin with a selenium atom had no significant influence on the observed cytotoxicity. The authors suggest that due to comparable cytotoxicity of selenodioscin and dioscin, similar molecular mechanisms, including the mitotic arrest of cell cycle and activation of caspase-3 and caspase-9, might contribute to the cytotoxicity exhibited by tested agents. Importantly, the authors suggest that further investigations might indicate some additional beneficial properties of novel organoselenium derivatives, including antioxidation and the ability to quench radicals [26].

Our recent study shows that novel derivatives of phenylselenides decrease the metabolic activity of MDR *Pseudomonas aeruginosa* and possess the ability to prevent biofilm-formation process [37]. Thus, the observed proprieties of the tested derivatives might aid in the development of effective strategies against MDR Gram-negative strains, especially *Pseudomonas aeruginosa*, which is directly associated with hospital infections *via* the colonization of medical devices, as well as a major cause of recurrence and chronic infection, such as pneumonia in cystic fibrosis patients and wound infections [64,65]. Moreover, due to the amphipathic nature of phenylselenides and their potential to be incorporated into into cell membranes and their structural similarity to analogues of antimicrobial peptides, where a wide range of activity has been confirmed, including anticancer activity, future research using these derivatives in the aspect of tumor treatment might produce promising results [66].

In the study performed by Fuentes-Aguilar et al. structure-activity relationships for the design of new compounds with organoselenium moiety for tackling cancer are analysed. Initially, their antiradical activity using the DPPH based method and GPx-like activity for the scavenging of peroxides in comparison to ascorbic acid, BHT and ebselen are evaluated. In both tests, results showed that selenoureas were indicated as the most potent compounds. In another set of experiments, the antiproliferative properties of natural steroids derivatives obtained from diosgenin, smilagenin, hecogenin, and estrone with organoselenium moiety against representatives of cell lines from mostly diagnosed cancers, including breast, cervix, lung, and colorectal were tested [44]. Results indicate that anticancer activity is strongly dependent on the nature of the organoselenium group, its position on the steroid and the substituents on the *N*-aryl fragments. Importantly, regarding ebselen activity, in most cases synthesized agents indicated higher antiproliferative activity. Furthermore, for the tested derivatives GI50 value was similar or lower if compared to widely used chemotherapeutic agents: 5-Fluorouracil and cisplatin, which suggest their significant potential as an anticancer agent. It should be emphasized that among all tested compounds selenoureas derivatives were noted as most active against all tested cell lines. The preliminary results of the perception of the mode of action showed that the low concentration of compound induced accumulation of cells in the G1phase of the cell cycle of breast cancer cell lines. Nevertheless, in the case of the effect on the remaining cell lines, a slight increase of the S compartment was observed. However, at higher concentrations cell death was observed in all cell lines.

In a report published by Cui et al. a family of selenadiazolylpregnenolone derivatives was synthesized and their *in vitro* activity against PC-3 (human prostate carcinoma), SKOV3 (human ovarian carcinoma), T47D (human breast infiltrating duct carcinoma), MCF-7(human breast adenocarcinoma) and HEK293 T (normal kidney epithelial cells) in comparison to abiraterone was performed [46]. The results indicated

that all pregnenolone selenadiazole compounds were compatible with normal kidney epithelial cells (HEK293T). Interestingly, for some derivatives selective antiproliferative activity against PC-3 and SKOV3 cell lines was observed. The strong inhibitory activity against human ovarian carcinoma was noted when the substituted group R is the alkyl group or when R is the thiophenyl or furanyl of 5-member heterocycle. In the case of human prostate carcinoma, derivatives with propyl, cyclopropyl or thiophenyl and furanyl moiety manifest good antiproliferative activity with IC50 value much higher than that noted for abiraterone. Of importance and worth further research is the fact that synthesized compounds do not exert inhibition activity against MCF-7 and T47D breast cancer cell lines.

It should be emphasized that ebselen is a selenoorganic compound which is part of the chemical library of the NIH Clinical Collection as a bioavailable drug considered clinically safe, but without proven use [67]. Various data indicate that ebselen is clinically safe for humans [68]. Apart from being approved for its ability to scavenge reactive oxygen species (ROS), ebselen has been described as a potent compound with cytotoxic activity against many kinds of human cancer cell lines [69,70]. Concerning its antioxidative activity, it has been reported that ebselen induced apoptosis through engagement of different mechanisms that involve intracellular thiol depletion, mitochondrial permeability transition as well as inhibition of ROS generation [71]. The aforementioned features might also explain its anti-inflammatory potential. Of note is the fact that several studies revealed that ebselen can reduce the side effects of many classical antitumor agents; while if applied in unison as a component of synergistic therapy might also enhance antitumor activity [72,73]. However, the data presented in Table 1 when ebselen activity was compared with steroid derivatives with organoselenium moiety, indicated better antiproliferative activity. For example, in the case of human cervical cancer cells, 14-fold greater activity for steroids with selenourea and selenocyanates moieties was observed when compared to ebselen activities. Based on the described activity, it could be postulated that their activity is associated with the connection of steroid activity with selenium properties. Thus, steroid derivatives with organoselenium moiety might seem to be attractive candidates for antitumor therapy. However, a further in-depth study is needed to explain their mode of action and potential use. The current state of knowledge indicates that this kind of compound has been only tested in an *in vitro* setting. The aforementioned limitation suggests that additional studies at an *in vivo* level should be performed for better characterization of this interesting class of compounds. Animal models are helpful in understanding the pharmacological properties of a compound, including its pharmacokinetic and pharmacodynamic properties and toxicological profile, and help to determine its suitability for use in the pharmaceutical industry. It is also encouraging that based on the proven pleiotropic activity of ebselen more studies should be performed to discover and understand the biomedical actions of steroid derivatives with organoselenium moiety related to health prevention and treatment of diseases. Nevertheless, we strongly believe that review of currently published studies will encourage scientists to carry them out.

4. Conclusions

In this review, several methods of hybrid synthesis formed by selenium moiety attachment to a steroid molecule were presented. In the 1980s and 1990s, selenosteroids were prepared as intermediates during syntheses of biologically active compounds or compounds useful for mechanistic studies. Recently, because selenium plays a key role as a redox modulator in protecting the cell against oxidative damage in living organisms, selenosteroids have been synthesized to check for their antioxidant and/or anti-cancer properties. Doubtless, currently published data indicate a huge potential for selenosteroids, particularly selenoures derivatives. However, further complex studies both *in vitro* and *in vivo* are necessary in order to introduce them for medical purposes.

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PhSeZnCl in the Synthesis of Steroidal β-Hydroxy-Phenylselenides Having Antibacterial Activity

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Abstract: We report here the reaction of in situ prepared PhSeZnCl with steroid derivatives having an epoxide as an electrophilic functionalization. The corresponding ring-opening reaction resulted to be regio- and stereoselective affording to novel phenylselenium-substituted steroids. Assessment of their antibacterial properties against multidrug-resistant bacteria, such as *Pseudomonas aeruginosa* Xen 5 strain, indicates an interesting bactericidal activity and their ability to prevent bacterial biofilm formation.

Keywords: steroids; selenium; antibacterial; antibiofilm

1. Introduction

The last few decades have seen a growing interest in the synthesis of organoselenium compounds due to some promising biological activities, reported in the recent literature [1–3]. Selenium is present in mammalian organisms in the form of selenoenzymes, embedded in the proteinogenic amino acid selenocysteine. All the selenoproteins demonstrated redox properties and have a crucial role in the redox modulation as well as in the control of the redox homeostasis of the living systems, playing also a role in the cell's protection against the oxidative stress [4]. The prooxidant activity of organoselenium compounds was recently described as an interesting property to be directed toward specific targets developing antibacterial [5], antiviral [6], and antifungal agents [7], hormetines [8], and enzyme inhibitors [9]. Furthermore, direct and indirect interferences with the redox homeostasis and the redox signaling can produce an anticancer effect affecting the differentiation, proliferation, senescence and death pathways in the cells [10].

Even if many physiological and pathological mechanisms involving organoselenium derivatives need to be clarified, the recent introduction of Ebselen® and Ebselen-like compounds in clinical trials for the treatment of diabetes complications and non-small cell lung cancer, further demonstrate the current interest in study these derivatives for medical purposes [11,12].

Till now, few examples of selenosteroids were reported in the literature and they were tested and proved to be interesting antiproliferative agents following a prooxidant mechanism.

Some representative examples are reported in Figure 1 [13–15], selenium can be embedded as selenourea, selenocyanate or alkyl/aryl selenide directly introduced in C-3 or C-6 through the S_N2 reaction of a selenolates with a tosylate (VI) or an epoxide (V) affording, in this letter case to a β-hydroxyselenide [16,17].

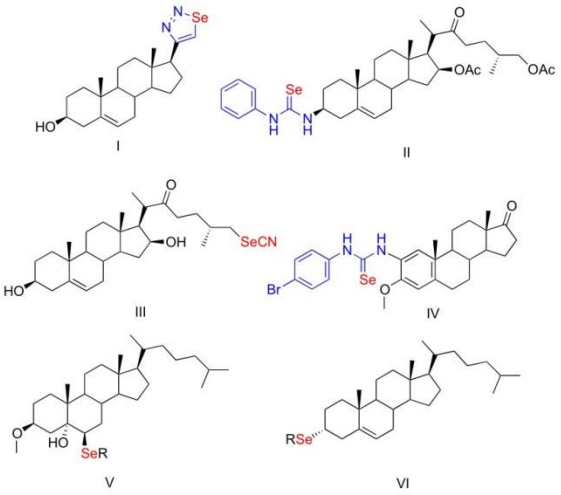


Figure 1. Some representative selenosteroids with antiproliferative and prooxidant activity. Selenium is directly bonded to the steroid (III, V and VI) selenium is imbedded in a different group (in blure) linked to the steroid (I, II, IV).

During the last ten years, some of us deeply investigated the use of nucleophilic selenium reagents, in the form of zinc selenolates, for the functionalization of electrophilic organic substrates [18]. Among these reagents the PhSeZnCl, easily prepared through the oxidative insertion of elemental zinc into Se-halogen bound of PhSeCl, it was the first bench stable organic selenolate [19], it is nowadays commercially available, and someone recently named as *Santi's* reagent [20].

These reagents demonstrate a broad range of applicability showing in several cases a strong rate acceleration when the reaction is performed in "on water" conditions [21–23].

In addition, its use was recently described also in the ring opening reactions of aziridines [24] and epoxides for the optimization of a total synthesis [25] as well as in the functionalization of preformed polymers [26], demonstrating the effective applicability of the method also starting from polyfunctionalized substrates. The ring opening of epoxide with PhSeZnCl is generally highly regioselective and the presence of the zinc as Lewis acid was demonstrated to be in some cases important for the regioselective control of the reaction [19].

2. Results and Discussion

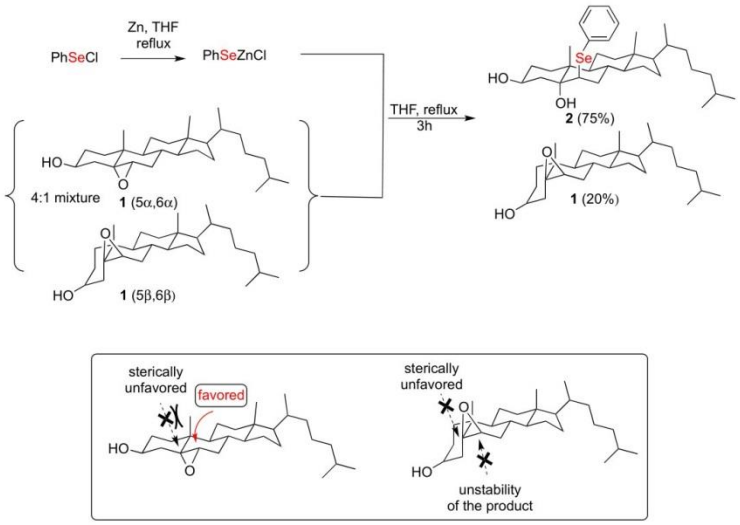
2.1. Chemistry

In the present work, we explored the use of PhSeZnCl for the functionalization of different steroid derivatives having an epoxide as an electrophilic reactive center, in order to obtain new molecular selenosteroids as prototypes for the investigation of the antibacterial activity. The optimization of the reaction conditions was performed starting from the epoxide **1** as a 4:1 mixture of the α and β isomer, respectively.

The reaction of 5,6-epoxycholestane **1** with solid PhSeZnCl was firstly investigated using the conditions reported for the opening of epoxide both in water suspension and THF solution at room temperature [19] but these procedures did not afford the desired product. Differently, when the reagent was prepared in situ in THF and the epoxide **1** was refluxed for 3 h with the reagent the β-hydroxyselenide **2** was obtained in 60% yield (75% on converted material), as depicted in Scheme 1. The nucleophilic attack to the epoxide ring occurs exclusively on the less sterically hindered carbon (C6) due to the presence of axial C-19 methyl group, indicating that an S_N2 mechanism is involved in the process and affording the corresponding *trans*-hydroxy selenides **2**. Only the *trans* hydroxyl selenide **2** was observed and the unreacted 5β,6β-epoxide was quantitatively recovered after chromatographic purification. The reason of the non-reactivity of 5β,6β-epoxide with nucleophilic reagents was recently explained and deal with the unfavorable formation of a constrained structure that should arise from the *trans*-diaxial opening at C6 with the hydroxyl at C5 in the *syn* position with regard to the C19 [27]. A separate experiment with epoxycholestane **1** and $(PhSe)_2$ in the presence of $NaBH_4$ was performed. After 3 h, TLC monitoring showed the consumption of a part of the starting material **1**. On the basis of ^1H NMR spectral analysis, it was established that only the α-epoxide **1** reacted while the β-isomer of **1** remained intact. Moreover, after 3 h of the reaction, 3β-hydroxy-5α,6α-epoxycholestane (20%) was still present in the reaction mixture.

Compound **2** was fully characterized by NMR, IR and MS spectroscopies and the collected data correspond to those previously reported by Rodrigues et al. [16].

In the optimized protocol, the zinc (1.0 equivalent) activated by treatment with HCl 10%, was added to the solution of PhSeCl (1.0 equivalent) in anhydrous THF and heated at the reflux temperature till the discoloration of the solution that is assumed to be indicative for the formation in situ of PhSeZnCl. At this point, the substrate **1** (1.0 equiv) dissolved in THF was added. The reaction was refluxed for an additional 3 h and monitored by thin-layer chromatography (TLC).



Scheme 1. Reaction of 3β-hydroxy-5α,6α-epoxycholestane (**1**) with in situ formed PhSeZnCl.

Using the above-described conditions, the scope of epoxy substrates **1, 3, 5, 6** and **8** was explored. The results are collected in Table 1. Interestingly the mild conditions of the procedure resulted compatible with the sensitive spiroacetal system [28] in **3** and the sterically hindered

epoxide **5** [29] was totally unreactive. The reactions of 1,2-epoxysteroids **6** and **8** with the in situ generated organoselenium reagent gave 1β,3β-dihydroxy-2β-phenylselenylcholestane (**7**) and 2β,3α-dihydroxy-1α-phenylselenylcholestane (**9**), respectively. For the accurate determination of the stereochemistry of the centers formed in the C-1 and C-2 positions, two-dimensional NMR experiments were performed (COrrelation SpectrocpY (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC)) in 400 MHz instrument. For compound **7**, the coupling system inferred from the correlation spectra indicates that phenylselenyl group is bonded to C-2, since 2-H is coupled to both adjacent 1-H and 3-H. In addition, the 3-H signal has the width at half-height of 24 Hz, which clearly indicates that it is axial, and therefore, 2-H must be equatorial because its width at half-height is small (10 Hz). The downfield shift of C-1 (78.4) shows that the configuration of the OH group is β (axial), and therefore, 1-H is equatorial. The correlation pattern inferred from COSY, HSQC, and HMBC spectra of compound **9** allows us to state that phenylselenyl group is bonded to C-1. Proton 3-H clearly has configuration β (equatorial) since its width at half-height is 8 Hz. In turn, 2-H has configuration α (equatorial) since long range coupling to 4-H_{eq} is observed in COSY spectrum filling the marks of the H-C-C-C-H W-shape structure (Figure 2) [30].

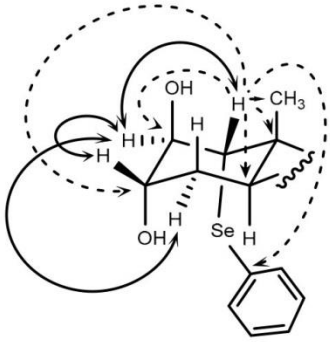


Figure 2. Relevant data from COrrelation SpectrocpY (COSY and heteronuclear multiple bond correlation (HMBC) experiments for the structure of the compound **9** (COSY correlations – plain line, HMBC correlations–dashed line, – means no relation).

Table 1. Scope of the reaction

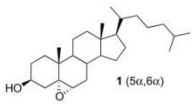
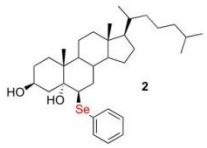
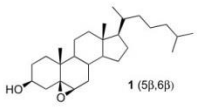
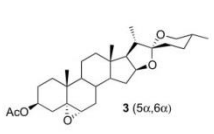
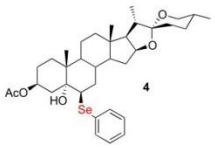
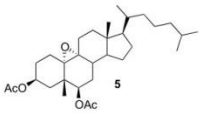
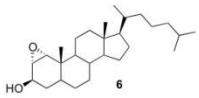
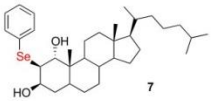
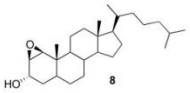
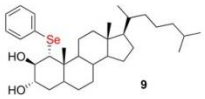
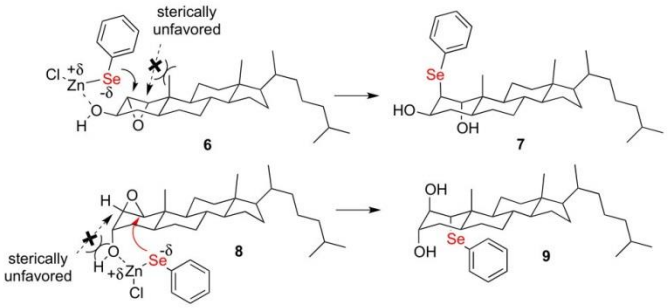
Entry	Substrate	Time (h)	Product	Yield
1	 **1** (5α,6α)	3	 **2**	75%
2	 **1** (5β,6β)	3	–	–

Table 1. *Cont.*

Entry	Substrate	Time (h)	Product	Yield
3	 **3** (5α,6α)	6	 **4**	62
4	 **5**	8	–	–
5	 **6**	2	 **7**	54
6	 **8**	2	 **9**	53

Based on these spectroscopic evidence, stereogenic centers at C-1 and C-2 have the absolute configuration depicted in structure **7** and **9**, and a plausible mechanism for their formation starting from 1,2-epoxysteroids **6** and **8** with PhSeZnCl is proposed in Scheme 2. In both cases, the nucleophilic attack proceeds in order to minimize the steric hindrance of the approaching selenium reagent with the substituents, following a pseudo-axial attack according to the Fürst-Plattner rule [31]. Furthermore, the interaction of zinc with the hydroxyl group at C-3 could increase the selenium nucleophilicity and cooperate on driving the observed regioselectivity. Steric factors and the lack of a suitable pseudo-axial approach resulted to be particularly detrimental in the reactivity of PhSeZnCl with the epoxy-steroids and could explain all the observed failures.



Scheme 2. The proposed mechanism for ring opening of **6** and **8** according to a pseudo-axial ring opening approach.

2.2. Biological Activity

A further purpose of this study was to investigate the biological properties of the prepared selenosteroids. The search for new compounds that exhibit antimicrobial properties is a big challenge

and an emerging need in modern medicine, especially in the context of the growing number of infections caused by multidrug-resistant bacteria. Furthermore, the antibacterial activity against the biofilm formation of *Pseudomonas aeruginosa*, which usually causes opportunistic infections, particularly in immunocompromised patients, can be considered a promising characteristic for the development of a new class of antibiotics [32].

The changes of *Pseudomonas aeruginosa*, Xen 5 luminescence provide an easy way to assess bacteria cell viability and metabolic function. [33] Accordingly, Figure 3A–E shows that all tested agents affect the viability of employed bacteria strain. However, activity of compound **7** was stronger when compared to other tested agents. More than 95% of decrease in *P. aeruginosa* Xen 5 chemiluminescence, was observed after 10 min of incubation when highest dose of **7** was tested. This inhibitory effect is comparable to activity of standard antibiotic agents used in concentrations corresponding to a 100-fold increase of MIC value (Figure 3E). The obtained results suggest that the position of phenylselenyl group might affect and modulate the antimicrobial activity. Additionally, the activity of synthesized compounds, was compared to that of colistin, currently used in the treatment of *P. aeruginosa* infections. Interestingly, it was observed that the use of colistin at a dose corresponding to 1xMIC value does not affect the metabolic function of *P. aeruginosa* infections caused by MDR strains. In turn, application of 5-fold MIC concentration disturbs the cell function at 60%.

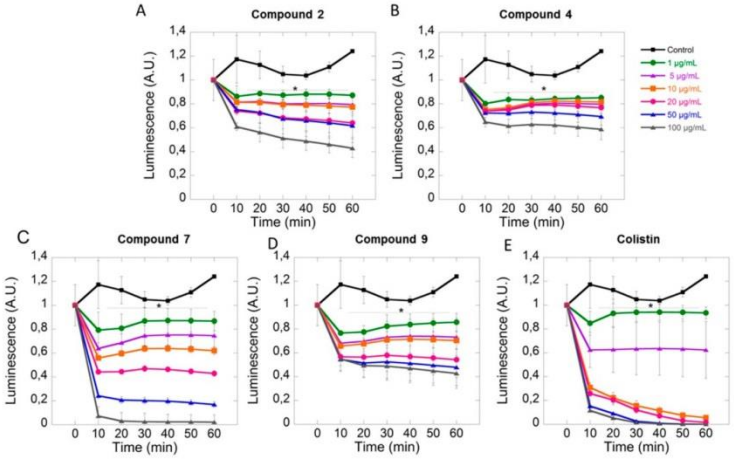


Figure 3. Phenylselenium-substituted steroids decrease the metabolic activity of MDR *Pseudomonas aeruginosa* strain. Panels **A**–**D** show activity of tested agents against planktonic form of *P. aeruginosa* Xen 5 in comparison to colistin (**E**). Statistical significance for the samples treated by tested compounds compared to control was marked by (*), $p \leq 0.05$. Results from 3 measurements ±SD.

In a different set of experiments, luminometric measurements was applied to determine the ability of the tested phenylselenium-substituted steroids on preventing bacterial biofilm formation. Figure 4A–C illustrates that the tested agents are able to inhibit biofilm formation and effectively kill bacteria embedded into the biofilm matrix in time and dose-dependent manners. However, after 24 h, in the case of compound **7**, high doses (>50 µg/mL) were required to obtain ~50% inhibition of biofilm formation. In the case of mature biofilm, formed after 48 and 72 h treatment with the tested agents (> 20 µg/mL), decreases biofilm viability by ~50%, for compound **2** and **4** respectively, and ~ 90% for compound **7**. In the case of colistin, their use in 1 and 5- fold MIC was insufficient to restrict biofilm metabolic activity. Therefore, the observed proprieties of the tested agents might help in developing of effective strategies against *Pseudomonas aeruginosa* biofilm formation, which is directly associated with

hospital infections via the colonization of medical devices, as well as a major cause of the recurrence and chronic infections, such as pneumonia in cystic fibrosis patients. Due to amphipathic nature of tested phenylselenium-substituted steroids and their similarity to ceragenins, which possess a broad spectrum of antimicrobial activity, further studies are needed to determine a full antimicrobial spectrum of the tested agents and to establish their mode of action [34].

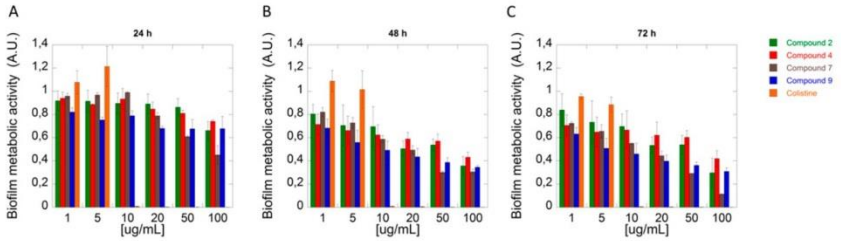


Figure 4. Anti-biofilm activity of phenylselenium-substituted steroids against *P. aeruginosa* strain. Ability of phenylselenium-substituted steroids to prevent biofilm-formation of *P. aeruginosa* after 24, 48 and 72 h. Results from 3 measurements ±SD.

The analysis of ^{77}Se-NMR chemical shift of **2**,**4**,**7** and **9** (reported in Figure 5) afforded interesting consideration. In compounds **7** and **9** the presence of hydroxyl groups in a suitable position to establish a non-bonding interaction with the selenium atom produce an evident upfield of the chemical shift (−60 ppm for **9**; −116 ppm for **7** respect **2** and **4**). This correspond to a higher electron density on the selenium atom of 7 respect to the other derivatives and, consequently, a lower electrophilicity to which correspond a reduced prooxidant activity and, reasonably, a lower general toxicity.

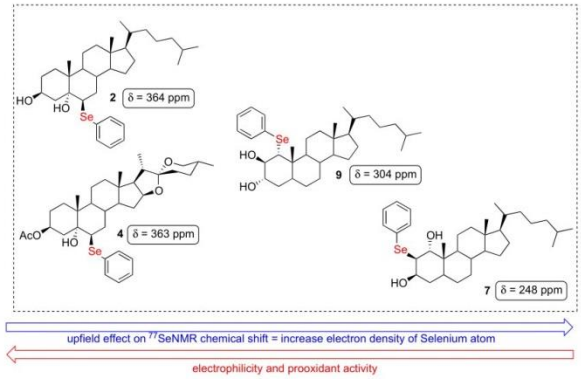
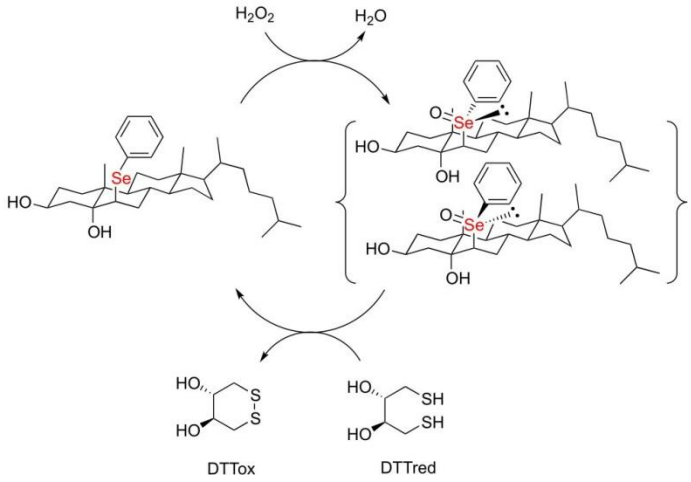


Figure 5. ^{77}SeNMR Chemical shift as parameter to predict biological aspects.

Considering that Rodrigues et al. reported that derivatives similar to **2** are characterized by an important prooxidant activity we explored the redox behavior of **2** as well as its GPx-like activity using the ^{77}Se-NMR and NMR-DTT coupled test [16,17]. Initially, we investigated by ^{77}Se-NMR spectroscopy, the actual intermediates involved in GPx-like cycle. With the addition of hydrogen peroxide (5 eq), we observed the formation of a couple of diastereomeric selenoxides (δ ^{77}Se-NMR in CD_3OD: 901 and 866 ppm), that can be readily reduced to selenide by the addition of a stoichiometric amount (5 eq) of reduced dithiotreitol (DTTred). The experiment was performed directly into the NMR tube (Scheme 3). The kinetics of the peroxide reduction catalyzed by **2** was measured studying via

^{1}H-NMR the oxidation of DTT. This process resulted particularly slow producing, after 19 h, only 22% of oxidized DTT. This value is particularly low respect those reported in literature for other GPx mimetics and we believe that it could be due to the steric hindrance around the selenium atom of selenoxide, that prevented the fast attack of the thiol and consequently the reduction of the selenoxide.



Scheme 3. Proposed redox cycle of 3β,5α-dihydroxy-6β-phenylselenylcholestane (**2**).

3. Materials and Methods

3.1. Chemistry

3.1.1. General Methods

Reagent-grade chemicals were purchased and used as received. Methylene chloride was freshly distilled. Flash column chromatography and flash chromatography were performed with silica gel, pore size 40A (70–230 mesh), unless otherwise stated. All reactions were monitored by TLC on silica gel plates 60 F_{254}. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra for all compounds were recorded at ambient temperature and were referenced to TMS (0.0 ppm) and $CDCl_3$ (77.0 ppm), respectively, unless otherwise noted. NMR resonance multiplicities were reported using the following abbreviations: b = broad, s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet; coupling constants *J* were reported in Hz. IR spectra were obtained in a $CHCl_3$ solution with an FT-IR spectrometer, and data are reported in cm^{-1}. Melting points were determined by a Kofler bench (Boetius type) apparatus and are uncorrected. (Spectra of the synthetized compounds are collected in the Supplementary Materials)

3.1.2. General Procedure

To a solution of the PhSeCl (1.0 equiv.) in anhydrous THF (1 mL/mmol) under argon atmosphere and at reflux, the activated zinc (1.0 equiv) was added. The formation of PhSeZnCl was indicated by the formation of colourless solution. Then, a solution of the starting material (1.0 equiv) in THF under argon atmosphere was added. The reaction was stirred at reflux for 3–8 h. Then THF was removed under vacuum. The yellow oil obtained was dissolved in CH_2Cl_2 and washed 3 times with H_2O. The organic layers were dried with Na_2SO_4, filtered and the solvent removed under vacuum. The products were purified by flash chromatography.

β,5*α*-Dihydroxy-6*β*-Phenylselenylcholestane (**2**)

The reaction with 3β-hydroxy-5ζ,6ζ-epoxycholestane [35] (1, 4*α*:1β, 100 mg, 0.2 mmol) was carried out (reaction time 3 h). Silica gel column chromatography gave pure compound **2** as a white solid (69 mg; 60%) eluted with ethyl acetate/hexane 1:4, and 3β-hydroxy-5β,6β-epoxycholestane was recovered. **2**: m.p. 159–162 °C (CH_2Cl_2/hexane). IR ν_{max} (cm^{-1}): 3554, 3407, 1064. ^{1}H NMR δ 7.56 (m, 2H), 7.27 (m, 3H), 4.08 (m, 1H), 3.10 (m, 1H), 2.46 (dd, *J* = 11.2, 13.3 Hz, 1H), 1.18 (s, 3H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 6.6 Hz, 3H), 0.74 (s, 3H). ^{13}C NMR δ 134.0 (CH × 2), 132.6 (C), 129.1 (CH × 2), 129.1 (CH), 78.1 (C), 68.1 (CH), 56.3 (CH), 55.5 (CH), 54.2 (CH), 46.1 (CH), 55.7 (CH), 45.4 (CH), 44.2 (CH_2), 42.8 (C), 39.9 (CH_2), 39.5 (CH_2), 39.3 (C), 36.2 (CH_2), 35.8 (CH), 34.5 (CH_2), 32.6 (CH_2), 31.3 (CH_2), 30.8 (CH_2), 28.2 (CH_2), 28.0 (CH), 24.2 (CH_2), 23.8 (CH_2), 22.8 (CH_3), 22.5 (CH_3), 21.3 (CH_2), 18.6 (CH_3), 17.6 (CH_3), 12.2 (CH_3). ^{77}Se NMR δ 365.8. HRMS calcd. for $C_{33}H_{52}O_2Se$: 560.3133, found: 560.3120.

(25R)-5*α*-Hydroxy-6*β*-Phenylselenylspirostan-3*β*-ol Acetate (**4**)

The reaction with (25*R*)-5α,6α-epoxyspirostan-3β-ol acetate [36] (3, 85 mg, 0.2 mmol) was carried out (reaction time 6 h). Silica gel column chromatography gave pure compound **4** as a white solid (70 mg; 62%) eluted with ethyl acetate/hexane 5:95. Colorless crystals: m.p. 270–273 °C (CH_2Cl_2/hexane). IR, ν_{max} (cm^{-1}) 3427, 3005, 1710, 1064. ^{1}H NMR: 7.54 (m, 2H), 7.27 (m, 3H), 5.15 (m, 1H), 4.39 (m, 1H), 3.47 (dd, *J* = 2.6, 9.4 Hz, 1H), 3.37 (m, *J* = 10.9 Hz, 1H), 3.06 (m, 1H), 2.47 (dd, *J* = 11.3, 13.4 Hz), 2.05 (s, 3H), 1.20 (s, 3H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.85 (s, 3H). 0.79 (d, *J* = 6.3 Hz, 3H); δ ^{13}C NMR: δ 171.8 (C), 134.7 (CH × 2), 131.9 (C), 129.1 (CH × 2), 127.1 (CH), 109.2 (C), 80.7 (CH), 71.2 (CH), 66.8 (CH_2), 62.2 (CH), 55.2 (CH), 54.0 (CH), 45.7 (CH), 40.8 (C), 40.4 (CH_2), 39.9 (CH_2), 39.5 (C), 36.6 (CH_2), 33.9 (CH_2), 32.3 (CH_2), 31.7 (CH_2), 31.4 (CH_2), 30.8 (CH), 30.3 (CH), 28.8 (CH_2), 26.6 (CH_2), 21.4 (CH_3), 21.0 (CH_2), 17.5 (CH_3), 17.1 (CH_3), 16.6 (CH_3), 14.5 (CH_3). ^{77}Se NMR δ 363.1. HRMS calcd. for $[C_{35}H_{50}O_5SeH]^+$: 631.2902, found: 631.2877.

1*α*,3*β*-Dihydroxy-2*β*-Phenylselenylcholestane (**7**)

The reaction with 3β-hydroxy-1α,2α-epoxycholestane [37] (**6**) (80 mg; 0.2 mmol) was carried out for 2 h. Silica gel column chromatography gave pure compound **7** eluted with ethyl acetate/hexane 15:85 as oil (60 mg; 54%). IR ν_{max} (cm^{-1}): 3429, 3055, 1458. ^{1}H NMR δ 7.64 (m, 2H), 7.26 (m, 3H), 4.39 (s, 1H), 4.09 (m, 1H), 3.78 (t, *J* = 2.5 Hz, 1H), 2.34 (d, *J* = 11.2 Hz, 1H), 0.96 (s, 3H), 0.91 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 6.6 Hz, 3H), 0.86 (d, *J* = 6.6 Hz, 3H), 0.67 (s, 3H). ^{13}C NMR δ 132.8 (C), 132.7 (CH × 2), 129.2 (CH × 2), 127.3 (CH), 78.4 (CH), 67.4 (CH), 59.5 (CH), 56.3 (CH × 2), 47.7 (CH), 42.6 (C), 40.1 (C), 39.8 (CH_2), 39.5 (CH_2), 39.0 (CH), 36.1 (CH_2), 35.9 (CH_2), 35.7 (CH), 34.9 (CH), 31.5 (CH_2), 28.2 (CH_2), 28.0 (CH_2), 27.9 (CH), 24.2 (CH_2), 23.8 (CH_2), 22.8 (CH_3), 22.5 (CH_3), 20.9 (CH_2), 18.7 (CH_3), 13.5 (CH_3), 12.1 l(CH_3). ^{77}Se NMR δ 248.4 ppm. HRMS calcd. for $C_{33}H_{52}O_2Se$: 560.3133, found: 560.3121.

2*β*,3*α*-Dihydroxy-1*α*-Phenylselenylcholestane (**9**)

The reaction with 3α-hydroxy-1β,2β-epoxycholestane [38] (**8**) was carried out for 2 h. Silica gel column chromatography gave pure compound **9** eluted with ethyl acetate/hexane 1:4. as white solid (60 mg, 53%); m.p. 174–175 °C (CH_2Cl_2/hexane); IR ν_{max} (cm^{-1}): 3565, 3351, 1437. ^{1}H NMR δ, ppm 7.54 (m, 2H), 7.26 (m, 3H), 4.39 (m, 1H), 4.09 (s, 1H), 3.50 (d, *J* = 2.6 Hz, 1H), 2.47 (bs, 1H), 2.11 (bs, 1H), 1.19 (s, 3H), 0.91 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 6.6 Hz, 3H), 0.68 (s, 3H). ^{13}C NMR δ 133.8 (CH × 2), 130.2 (C), 129.2 (CH × 2), 127.4 (CH), 73.9 (CH), 67.9 (CH), 57.4 (CH), 56.2 (CH), 56.1 (C), 52.4 (CH), 42.6 (C), 41.3 (CH), 39.6 (CH_2), 39.5 (CH_2), 36.1 (CH_2), 35.7 (CH), 35.1 (CH), 32.9 (CH_2), 31.5 (CH_2), 28.7 (CH_2), 28.2 (CH_2), 27.9 (CH), 24.2 (CH_2), 23.8 (CH_2), 22.8 (CH_3), 22.5 (CH_3), 20.7 (CH_2), 18.6 (CH_3), 15.9 (CH_3), 12.1 (CH_3). ^{77}Se NMR δ 304.5 ppm. HRMS calcd. for $C_{33}H_{52}O_2Se$: 560.3133, found: 560.3139.

3.2. Biological Activity

3.2.1. Antibacterial Testing

To assess the biological activities of synthesized compounds, we have chosen to determine their antibacterial activity against Gram-negative, multidrug resistant (MDR) *Pseudomonas aeruginosa* Xen 5 strain. Briefly, a bacterial culture was grown to mid-log phase at 37 °C, re-suspended in LB, and brought to 10^8 CFU/mL. Then 100 μL of bacteria suspensions were added to tested agents at a concentration range 1–100 μg/mL. Colistin was use as a control, since it represents "last treatment option" in some infections caused by MDR *Pseudomonas aeruginosa*. Upon bacteria addition the chemiluminescence intensity were registered using Labsystems Varioscan Flash (Thermo Scientific Waltham, MA, USA) over a period of 1h.

3.2.2. Anti-Biofilm Activity

The biomass of biofilm, formed by *Pseudomonas aeruginosa* Xen 5 in the presence of tested molecules, was evaluated using a luminescence technique as described previously [39]. Chemiluminescence intensity of *Pseudomonas aeruginosa* Xen 5 biomass was measured after 24, 48, and 72 h of growth. In this setting, time-dependent biofilm mass and viability was determined

3.2.3. GPx-Like Activity by NMR

In a 5mm NMR tube containing 0.15 mmol of DTT^{red} and 0.015 of catalyst (**2**) dissolved in 0.55 mL of CD_3OD, 0.15 mmol of H_2O_2 (30%) was added and ^{1}H-NMR experiments at 200 MHz were recorded at regular interval for a period of 24 h. Ratios between DTT^{red}/DTT^{ox} were determined by comparison of integrals at 2.88 ppm and 2.64 ppm, respectively.

4. Conclusions

In conclusion we demonstrated that in situ formed PhSeZnCl can be used for the functionalization of steroids with selenium moieties. The obtained novel compounds were tested for their potential antibacterial properties against *Pseudomonas aeruginosa* Xen 5 strain, evidencing a promising bactericidal activity associated to a biofilm formation prevention.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/9/2121/s1.

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Abbreviations

COSY	COrrelation SpectrocpY
DTT	DiThioTreitol
GPx	Glutathione Peroxidase
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation

IR	InfraRed
MIC	Minimum Inhibitory Concentration
MS	Mass
NMR	Nuclear Magnetic Resonance
SD	Standard Deviation
THF	TetraHydroFuran
TLC	Thin Layer Chromatography

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Simple Zn-Mediated Seleno- and Thio-Functionalization of Steroids at C-1 Position

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Abstract: Here we report the reaction in the biphasic system of the in situ prepared selenols and thiols with 1,4-androstadiene-3,17-dione (**1**) or prednisone acetate (**2**) having α,β-unsaturated ketone as an electrophilic functionalization. The Michael-type addition reaction resulted to be chemo- and stereoselective, affording a series of novel steroidal selenides and sulfides. This is an example of a one-step, eco-friendly process that bypasses some of the main concerns connected with the bad smell and the toxicity of these seleno- and thio-reagents. Furthermore, we demonstrated that the proposed methodology offers the possibility to prepare libraries of steroids variously and selectively decorated with different organochalcogen moieties at the C1 position starting from 1,4-androstadienic skeletons and leaving unaltered the C4–C5 unsaturation. Based on the data reported in the literature the introduction of an organoselenium or an organosulfur moiety in a steroid could provide new interesting pharmaceutically active entities exerting anticancer and antimicrobial activities. In this optic, new synthetic strategies to efficiently prepare this class of compounds could be strongly desirable.

Keywords: selenium; sulfur; zinc; steroids; Michael additions

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1. Introduction

In the last decades different classes of organoselenium compounds were investigated for biological purposes, evidencing, besides the antioxidant properties [1], some promising activities, such as antiviral, antibacterial and anticancer [2,3].

Similarly, the cyclopenta[a]phenanthrene skeleton is a privileged core structure present in several pharmacologically relevant molecules as well as in some commercially available drugs and/or hormones such as glucocorticoids, steroidal anti-inflammatories or cardiac steroids [4]. On the basis of these considerations, a hybrid formed by placing a Se- or a S-moiety to a steroidal structure may have enhanced biological properties when compared to the native fragments [5]. Consequently, improving synthetic tools in order to enable the chemo regio- and stereoselective preparation of novel selenium- and sulfur-containing libraries of steroids is particularly challenging for the exploration of the chemical space in the discovery of novel biologically active compounds. Right now, a small number of selenosteroids are reported in the literature, and some general examples of functionalization on different carbons of the cyclopentanoperhydro-phenanthrene skeleton are summarized in Figure 1. Selenium can be contained in functionalized selenoureas [6], or heterocycles such as *N*-linked selenoxazoles [7] or 1,2,3-selenodiazoles [8] that are generally introduced

using multistep procedures. Ibrahim-Ouali in 2009 described the first total synthesis of 11-selenosteroids as the unique example in which a carbon of the steroidal skeleton is substituted with a selenium atom [9].

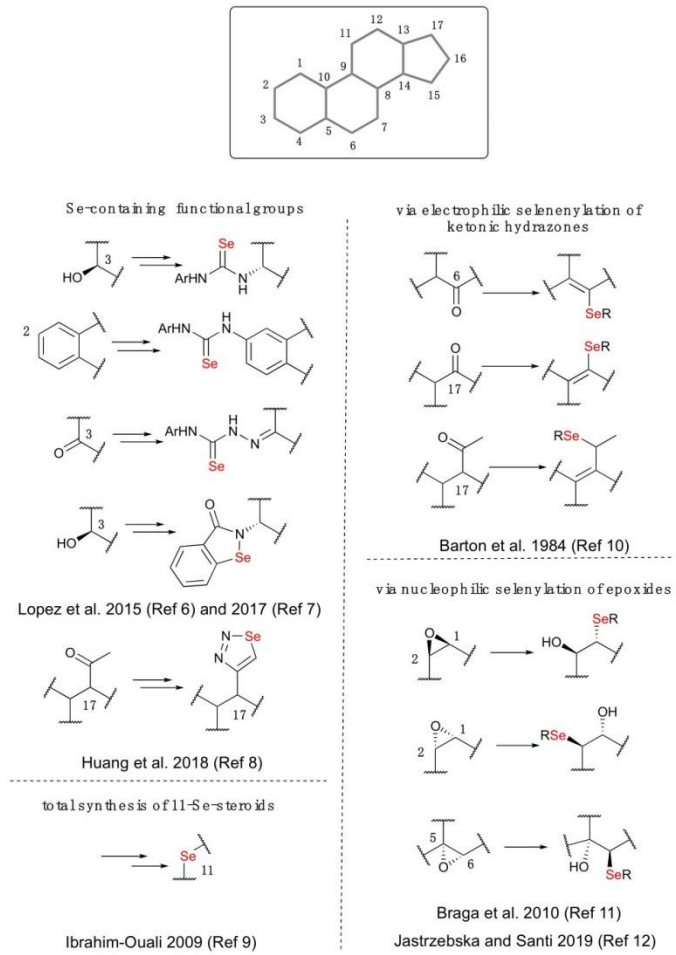


Figure 1. Examples of Se-functionalization on cyclopentanoperhydro-phenanthrene skeleton.

In other examples, electrophilic and or nucleophilic selenium reagents were directly introduced in the structures using the reactivity of the ketonic hydrazones [10] and of the epoxides, respectively [11].

More specifically, Barton reported the conversion of C-6 and C-17 keto groups into vinyl selenide steroidal systems [10], and Braga and coworkers prepared a series of seleno-cholestane derivatives by the stereoselective ring opening reaction of 5α,6α epoxide with selenolates, generated in situ by the $NaBH_4$-mediated reduction of diphenyldiselenide $(PhSe)_2$ and other, differently functionalized diselenides [11]. This chemistry was expanded at different positions within the steroidal core by some of us who recently reported the

epoxide transformation exploiting the reactivity of PhSeZnCl, obtaining selenosteroids endowed with antibiofilm activity [12,13].

With the aim to develop a novel procedure to prepare hybrid derivatives [5], we report here the functionalization of the steroidal core of the biologically relevant androstadiene and prednisone bearing an α β-unsaturated keto system, which underwent Seleno-Michael or Thio-Michael addition by treatment with selenolates and thiolates generated in situ using a previously reported acidic biphasic system that was extensively used for the selenenylation of different classes of organic compounds [14–20].

2. Results and Discussion

For the current investigation we slightly modified the procedure recently reported by some of us for the conjugated nucleophilic addition of selenolates [20]. A biphasic system composed by the same volume of ethyl acetate and 10% HCl, containing $PhSe_2$ (**3a**) and 10 equiv. of zinc shaves was stirred until complete discoloration of the organic layer. The liquid phase was transferred under argon atmosphere into a flask containing the substrate: 1,4-androstadiene-3,17-dione (**1**) or prednisone acetate (**2**). The resulting reaction mixture was stirred at room temperature for 3 h (Figure 2). When compound **1** was used as starting material, the formation of steroidal selenide **4a** was regio- and stereoselectively obtained and isolated in 70% yield after chromatographic purification, having a physical and spectroscopic date fully coherent with those reported in the literature [21]. The selenenylation afforded only the α diastereisomer at C1 carbon as a consequence of the steric hindrance at the electrophilic carbons.

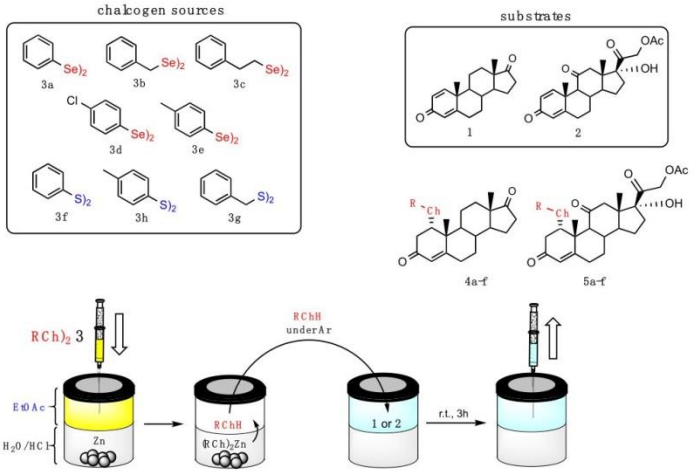


Figure 2. Addition reaction of nucleophilic reagents prepared in situ from the dichalcogenides **3a–g** to the Michael acceptors **1** and **2** affording the target compounds **4a–f** and **5a–g,** respectively (the scopes are reported in Tables 1–3).

Table 1. Seleno-Michael reactions on substrate (**1**).

1 **4a-e**

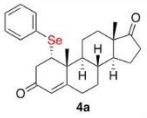
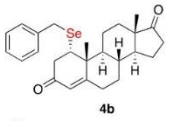
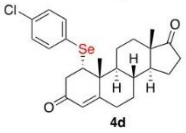
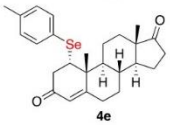
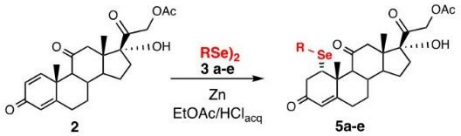
Entry	$(RSe)_2$ (3)	Product (4)	Yield %
1	**3a**	 **4a**	70
2	**3b**	 **4b**	48
3	**3c**	 **4c**	51
4	**3d**	 **4d**	81
5	**3e**	 **4e**	64

Table 2. Seleno-Michael reactions on substrate (**2**).



2 **5a-e**

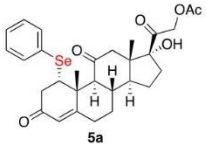
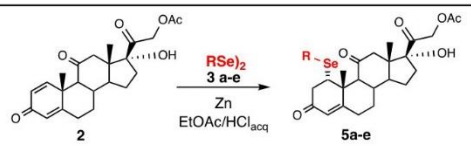
Entry	$(RSe)_2$ (3)	Product (5)	Yield %
1	**3a**	 **5a**	70

Table 2. *Cont.*



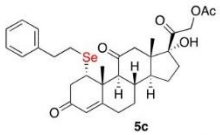
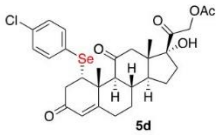
Entry	$(RSe)_2$ (3)	Product (5)	Yield %
2	**3b**	 **5b**	52
3	**3c**	 **5c**	69
4	**3d**	 **5d**	69
5	**3e**	 **5e**	96

Table 3. Thio-Michael reactions.

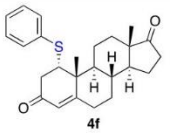
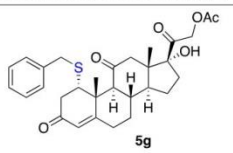
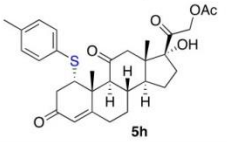
Entry	Substrate	$(RS)_2$ (3)	Product (4 from 1 or 5 from 2)	Yield %
1	**1**	**3f**	 **4f**	35
2	**2**	**3f**	 **5f**	33

Table 3. *Cont.*

Entry	Substrate	$(RS)_2$ (3)	Product (4 from 1 or 5 from 2)	Yield %
3	**2**	**3g**	 **5g**	11
4	**2**	**3h**	 **5h**	33

The scope of the reaction was investigated by the use of commercially available diselenide (**3a**) or diselenides prepared according to the literature (**3b**–**e**) [22]. Diphenyldiselenide (**3a**) and diaryldiselenides bearing both electron withdrawing (**3d**) or donating (**3e**) substituents afforded the corresponding selenenylated steroids **4a**, **4d** and **4e**, in good yields (Table 1 entries 1, 4 and 5). On the contrary, dibenzyldiselenide (**3b**) and bis(2-phenylethyl)diselenide (**3c**) gave the target compounds only in moderate yields (Table 1, entries 2 and 3). In all the cases the reactions resulted in being regio- and stereoselective, as described for the conversion of **1** into **4a**.

The same panel of diselenides (**3a**–**e**) were reacted with prednisone acetate (**2**), which is the prodrug of prednisolone, a widely used steroidal anti-inflammatory drug [23]. As depicted in Table 2, the reactivity resulted to be very similar to that observed for 1,4-androstadiene-3,17-dione (**1**).

The C1-α-selenenylated derivatives **5a**–**e** were obtained in isolated yields ranging from 52% to 96% (Table 2). Interestingly the ester functionality resulted in being compatible with the applied conditions, and it was not affected by the aqueous acidic conditions.

By using the same protocol, the substrates **1** and **2**, and disulfides **3f**–**h** as chalcogenate sources, the scope of the Thio-Michael **addition** was explored. The results reported in Table 3 were obtained by reducing the commercially available, colorless disulfides **3f**–**h** for 15 min in the zinc-containing, biphasic acidic system [20]. Then, organic and aqueous layers were transferred under argon into a flask containing 1,4-androstadiene-3,17-dione (**1**) or prednisone acetate (**2**), and the resulting mixture was stirred for an additional 3 h at room temperature. As a result of the reduced nucleophilicity of the sulfur atom, thioderivatives **4f** and **5f**–**h** were obtained in lower yields when compared to the selenium analogues, but with the same regio- and stereoselectivity, indicating a lower reactivity of sulfur when compared to selenium in the tested conditions.

3. Conclusions

In conclusion, we developed a new methodology for the regio- and stereoselective synthesis of seleno- and thiosteroids using chalcogenating reagents generated in situ by the Zn-mediated reduction of diselenides or disulfides in a biphasic acidic medium. The resulting chalcogen cantered nucleophiles were reacted with model steroids having a Michael acceptor functionalization, affording the target compounds in poor to excellent yields after chromatographic purification.

4. Experimental Methods

4.1. General Information

Solvent reagents and commercially available starting materials were purchased from Sigma-Aldrich (St. Louis, MO, USA), Alfa Aesar (Kandel, Germany), and VWR (Milano,

Italy), and used as received unless otherwise noted. Diselenides **3b–e** were synthesized as reported in the literature [22]; the physical and spectral data of **4a–f** and **5a–h** are reported below and all the spectra are reported in the Supporting Information. Reactions were conducted in round-bottom flasks and were stirred with Teflon coated magnetic stirring bars (Sigma-Aldrich, St. Louis, MO, USA). Flash chromatography was performed with silica gel, pore size 40A (70–230 mesh) unless otherwise stated. All reactions were monitored by TLC on silica gel plates 60 F254 (Merck, Darmstadt, Germany). NMR experiments were performed in a Bruker Advance 400 spectrometer (Bruker, Fällanden, Switzerland). Only selected signals in the ^{1}H NMR spectra are reported. The ^{1}H and ^{13}C NMR chemical shifts (δ) are reported in parts per million (ppm), and they are relative to TMS (0.0 ppm) and the residual solvent peak ($CDCl_3$, 7.27 for ^{1}H NMR, and 77.0 ppm for ^{13}C NMR). The ^{77}Se chemical shifts (δ) are reported in parts per million (ppm), and they are relative to diphenyl diselenide (464 ppm) in $CDCl_3$. Data are reported as follows: chemical shift, multiplicity, coupling constants, where applicable, and the number of hydrogen atoms. Abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublet), dt (doublet of triplet), tt (triplet of triplet), m (multiplet), br.s. (broad signal). Coupling constant (J) is quoted in Hz to the nearest 0.1 Hz. High-resolution mass spectrometry (HRMS) measurements were performed using an Agilent 6520 QTOF instrument (Agilent, Santa Clara, CA, USA). IR spectra were obtained in a $CHCl_3$ solution with a Thermo Scientific, Nicolet 6700 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA) and data are reported in reciprocal centimeters. Melting points were determined by a Kofler bench (Boetius type) apparatus and are uncorrected (Wagner & Munz GmbH, Munchen, Germany).

4.2. General Procedure for the Michael-Type Addition

Diselenide or disulfide (1.3 equiv.) was added to a flask with 2 mL of 10% HCl, 2 mL of ethyl acetate, then 13 equiv. of zinc shaves (or turnings) were added. The reaction was stirred vigorously (800 rpm) until the discoloration of the organic layer occurred (15–20 min), in the case of colorless disulfides, the reaction was kept for 15 min. Then, the biphasic mixture was separated by the unreacted zinc and transferred under inert conditions (Ar) into a vial containing the steroid 1 or 2 (1 equiv) using the double-ended cannula technique. The reaction mixture was stirred for 3 h at room temperature, poured into water and extracted with ethyl acetate (3 × 20 mL). The organic layer was dried with Na_2SO_4, filtered and the solvent removed under vacuum. The products were purified by flash chromatography (Figures S1–S64).

1α-phenylselenylandrost-4-en-3,17-dione (**4a**) [21]

Isolated as a white solid after flash chromatography, eluent petroleum ether/ethyl acetate (7:3). Yield 70%. m.p. (CH_2Cl_2/hexane): 172–174 °C [21]: 192.1–193.8 °C); IR,υ_{max} (cm^{-1}) 1736, 1677, 1479; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.49–7.47 (m, 2H, *o*-C*H*-Ar), 7.24–7.20 (m, 3H, C*H*-Ar), 5.77 (s, 1H, C*H*=C), 3.55 (m, 1H, C*H*-Se), 2.94 (d, 1H, *J* = 17 Hz, C*H*H), 2.60 (d, 1H, *J* = 17 Hz, CH*H*), 1.31 (s, 3H, CH_3), 0.87 (s, 3H, CH_3) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$, 298 K, TMS): δ 220.3, 196.4, 166.2, 135.9, 129.4, 128.6, 128.4, 124.7, 51.4, 50.7, 50.2, 47.5, 43.0, 40.7, 35.8, 35.3, 32.4, 31.0, 29.7, 21.8, 19.7, 19.1, 13.8 ppm; ^{77}Se NMR (76.3 MHz, $CDCl_3$, 298 K, TMS): δ 345.9 ppm. HRMS calculated for $C_{25}H_{31}O_2Se$ 443.1484, found 443.1494.

1α-benzylselenylandrost-4-en-3,17-dione (**4b**)

Isolated as a white solid after flash chromatography using petroleum ether/ethyl acetate (7:3); 48% of yield. m.p. (CH_2Cl_2/hexane): 153–155 °C; IR,υ_{max} (cm^{-1}) 2958, 1736, 1654, 1157; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.23–7.14 (m, 5H, C*H*-Ar), 5.69 (s, 1H, C*H*=C), 3.76 (d, 1H, *J* = 12.3Hz), 3.56 (d, 1H, *J* = 12.3 Hz), 3.09 (dd, 1H, *J* = 3.3 and 16.9 Hz), 2.93 (m, 1H, C*H*Se), 2.76 (dd, 1H, *J* = 2.2 and 16.9 Hz), 1.18 (s, 3H, CH_3), 0.77 (s, 3H, CH_3) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$, 298 K, TMS): δ 220.6, 196.6, 167.4, 138.6, 129.0, 128.5, 127.0, 124.6, 50.7, 50.0, 47.5, 45.3, 42.5, 41.2, 35.8, 35.2, 32.3, 31.0, 29.6, 27.1, 21.7, 18.9, 18.6,

13.7 ppm; ^{77}Se NMR (76.3 MHz, $CDCl_3$, 298 K, TMS): δ 311.6 ppm. HRMS calculated for $C_{26}H_{33}O_2Se$ 457.1640, found 457.1652.

1α-phenylethylselenylandrost-4-en-3,17-dione (**4c**)

Isolated as a white solid after flash chromatography using petroleum ether/ethyl acetate (6:4); 51% yield. m.p. (CH_2Cl_2/hexane): 163–165 °C; IR,υ_{max} (cm^{-1}), 1744, 1243, 1187; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.23–7.20 (m, 2H, C*H*-Ar), 7.16–7.10 (m, 3H, C*H*-Ar), 5.71 (s, 1H, C*H*=C), 3.25 (m, 1H, C*H*-Se), 3.07 (dd, 1H, *J* = 3.5 and 17 Hz), 2.95–2.75 (m, 2H), 1.32 (s, 3H, CH_3), 0.88 (s, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 220.4, 196.5, 166.6, 155.4, 140.8, 128.5, 128.4, 127.7, 126.5, 124.5, 50.6, 50.0, 47.5, 45.8, 42.8, 41.0, 36.7, 35.8, 35.3, 32.4, 31.2, 31.0, 25.0, 21.8, 19.6, 18.9, 13.7 ppm; ^{77}Se NMR (76.3 MHz, $CDCl_3$, 298 K, TMS): δ 215.4 ppm. HRMS calculated for $C_{27}H_{35}O_2Se$ 471.1797, found 471.1808.

1α-(4-chlorophenylselenyl)-androst-4-en-3,17-dione (**4d**)

Isolated as a white solid after flash chromatography using petroleum ether/ethyl acetate (6:4); 81% of yield. m.p. (CH_2Cl_2/hexane): 175–177 °C; IR,υ_{max} (cm^{-1}) 2847, 1738, 1663, 1471; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.45 (d, *J* = 8.4 Hz, 2H), 7.24 (d, *J* = 8.4 Hz, 2H), 5.81 (s, 1H), 3.60 (m, 1H), 2.99 (dd, *J* = 3.4 and 17.2 Hz, 1H), 2.61–2.40 (m, 4H), 2.16–2.07 (m, 1H), 2.04–1.30 (m, 11H), 1.24–1.10 (m, 2H), 0.93–0.87 (m, 4H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 220.2, 196.1, 166.0, 137.3, 134.9, 129.6, 124.6, 51.6, 50.6, 50.2, 47.4, 43.0, 40.5, 35.8, 35.3, 32.3, 31.0, 29.7, 21.8, 19.7, 19.1, 13.8 ppm; ^{77}Se NMR (76.3 MHz, $CDCl_3$, 298 K, TMS): δ 339.4 ppm. HRMS calculated for $C_{22}H_{30}ClO_2Se$ 477.1094, found 477.1084.

1α-(4-methylophenylselenyl)-androst-4-en-3,17-dione (**4e**)

Isolated as a white solid after flash chromatography using petroleum ether/ethyl acetate (6:4); 64% of yield. m.p. (CH_2Cl_2/hexane): 182–184 °C; IR,υ_{max} (cm^{-1}) 2920, 1729, 1673, 1187; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.43 (d, *J* = 7.9 Hz, 2H), 7.09 (d, *J* = 7.8 Hz, 2H), 5.82 (s, 1H), 3.55 (m, 1H), 2.97 (dd, *J* = 17.2 and 3.5 Hz, 1H), 2.65 (dd, *J* = 17.1 and 2.5 Hz, 1H), 2.33 (s, 3H, CH3), 1.36 (s, 3H, CH3), 0.93 (s, 3H, CH3) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 220.2, 196.4, 166.1, 138.4, 136.1, 130.1, 124.8, 124.6, 51.4, 50.6, 50.0, 47.4, 42.9, 40.5, 35.7, 35.3, 32.3, 30.9, 29.6, 21.7, 21.2, 19.6, 19.0, 13.7 ppm; ^{77}Se NMR (76.3 MHz, $CDCl_3$, 298 K, TMS): δ 335.7 ppm. HRMS calculated for $C_{26}H_{33}O_2Se$ 457.1640, found 457.1627.

1α-phenyltioandrost-4-en-3,17-dione (**4f**) [21]

Isolated as a white solid after flash chromatography using petroleum ether/ethyl acetate (6:4); 35% of yield. m.p. (CH_2Cl_2/hexane): 186–190 °C (ref[21] 188.1–189.3 °C); IR,υ_{max} (cm^{-1}): 1740, 1685, 1613, 1475. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.41–7.39 (m, 2H), 7.31–7.27 (m, 3H), 5.84 (s, 1H, C*H*=C), 3.55 (m, 1H, C*H*-S), 2.77 (dd, 1H, *J* = 3.0 and 16.9 Hz), 1.38 (s, 3H, CH_3), 0.94 (s, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 220.4, 196.2, 165.7, 133.9, 133.7, 129.3, 128.0, 124.6, 54.4, 50.7, 47.9, 47.5, 42.7, 39.7, 35.8, 35.2, 32.4, 31.0, 29.7, 21.8, 19.9, 19.6, 13.8 ppm. HRMS calculated for $C_{25}H_{31}O_2S$ 395.2039, found 395.2056.

1α-phenylselenyl-17,21-dihydroxy-pregn-4-eno-3,12,20–trioxo–21–acetate (**5a**)

Isolated as a white solid after flash chromatography, eluent petroleum ether/ethyl acetate (6:4); 70% of yield. m.p. (CH_2Cl_2/hexane): 204–206 °C; IR,υ_{max} (cm^{-1}) 2973, 1692, 1654, 1433; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.42–7.40 (m, 2H, C*H*-Ar), 7.22–7.18 (m, 3H, C*H*-Ar), 5.72 (s, 1H, C*H*=C), 5.04 (d, 1H, *J* = 17.6 Hz), 4.66 (d, 1H, *J* = 17.6 Hz), 4.45–4.44 (m, 1H, C*H*Se), 3.10–2.90 (m, 2H), 2.80–2.20 (m, 8H), 2.10 (s, 3H, CH_3), 1.95–1.55 (m, 5H), 1.48 (s, 3H, CH_3), 1.45–1.20 (m, 3H), 0.62 (s, 3H, CH_3) ppm; ^{13}C NMR (100.6 MHz, $CDCl_3$, 298 K, TMS): δ 208.9, 204.6, 197.2, 170.6, 165.1, 135.4, 129.2, 128.5, 128.1, 124.8, 89.0, 67.7, 60.2, 51.8, 51.3, 49.6, 49.4, 42.7, 40.7, 36.8, 35.0, 32.0, 31.6, 23.2, 20.5, 18.4, 15.5 ppm. HRMS calcd for $C_{29}H_{35}O_6Se$ 559.1593, found 559.1599.

1α-benzylselenyl-17,21-dihydroxy-pregn-4-eno-3,12,20-trioxo-21-acetate (**5b**)

Isolated as a white solid, eluent petroleum ether/ethyl acetate (6:4); 52% of yield. m.p. (CH_2Cl_2/hexane): 175–177 °C; IR,υ_{max} (cm^{-1}) 2853, 1751, 1695, 1598; ^{1}H NMR

(400 MHz, $CDCl_3$, 298 K, TMS): δ 7.29–7.22 (m, 5H, C*H*-Ar), 5.74 (s, 1H, C*H*=C), 5.12 (d, 1H, *J* = 17.7 Hz), 4.71 (d, 1H, *J* = 17.6 Hz), 4.18 (m, 1H, C*H*Se), 3.75 (d, 1H, *J* = 11.5 Hz), 3.65 (d, 1H, *J* = 11.5 Hz), 2.20 (s, 3H, CH_3), 1.52 (s, 3H, CH_3), 0.67 (s, 3H, CH_3) ppm. ^{13}C-NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 209.1, 204.6, 197.2, 170.7, 166.1, 138.3, 128.9, 128.6, 126.9, 124.9, 89.1, 67.7, 60.0, 59.9, 51.5, 51.2, 49.6, 49.4, 47.0, 42.7, 42.4, 41.9, 36.8, 36.1, 32.6, 31.5, 28.8, 23.3, 20.6, 18.4, 15.5 ppm; ^{77}Se NMR (76.3 MHz, $CDCl_3$, 298 K, TMS): δ 295.7 ppm. HRMS calculated for $C_{30}H_{37}O_6Se$ 573.1750, found 573.1758.

1α-phenylethylselenyl-17,21-dihydroxy-pregn-4-eno-3,12,20-trioxo-21-acetate (**5c**)

Isolated as a white solid after flash chromatography using petroleum ether/ethyl acetate (6:4); 69% yield. m.p. (CH_2Cl_2/hexane): 203–205 °C; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): 7.31–7.29 (m, 2H, C*H*-Ar), 7.22–7.16 (m, 3H, C*H*-Ar), 5.74 (s 1H C*H*=C), 5.12 (d, 1H, *J* = 17.5 Hz), 4.71 (d, 1H, *J* = 17.5 Hz), 4.26 (m, 1H, C*H*Se), 3.27 (dd 1H *J* = 3.8 and 13.2 Hz), 2.17 (s 3H), 0.68 (s 3H); ^{13}C-NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 209.2, 204.4, 196.5, 170.5, 165.4, 140.9, 128.4, 126.3, 124.9, 89.0, 67.5, 60.0, 51.2, 49.6, 49.5, 46.7, 42.8, 42.0, 36.8, 36.7, 35.1, 32.1, 31.4, 29.7, 26.1, 23.3, 20.4, 18.4, 15.5; ^{77}Se NMR (76.3 MHz, $CDCl_3$, 298 K, TMS): δ 222.7; HRMS calculated for $C_{30}H_{37}O_6Se$ 587.1906, found 587.1910.

1α-(4-chlorophenylselenyl)-17,21-dihydroxy-pregn-4-eno-3,12,20-trioxo-21-acetate (**5d**)

Isolated as a white solid after flash chromatography using petroleum ether/ethyl acetate (6:4); 69% yield. m.p. (CH_2Cl_2/hexane): 207–209 °C; IR,υ_{max} (cm^{-1}) 2950, 1751, 1662, 1467; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.40 (d, *J* = 8.4 Hz, 2H, C*H*-Ar), 7.22 (d, *J* = 8.4 Hz, 2H, C*H*-Ar), 5.78 (s, 1H, C*H*=C), 5.10 (d, *J* = 17.6 Hz, 1H), 4.74 (d, *J* = 17.6 Hz, 1H), 4.50 (m, 1H, C*H*Se), 3.50 (br s, 1H, OH), 3.10 (dd, *J* = 3.6 and 17.2 Hz, 1H), 2.17 (s, 3H, CH_3), 1.54 (s, 3H, CH_3), 0.67 (s, 3H, CH_3) ppm; ^{13}C-NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 209.2, 204.7, 197.3, 170.7, 165.2, 136.7, 134.6, 129.4, 126.6, 124.7, 88.9, 67.8, 60.2, 52.2, 51.2, 49.6, 49.4, 42.7, 40.6, 36.8, 34.9, 32.0, 31.6, 23.2, 20.5, 18.3, 15.4 ppm; ^{77}Se NMR (76.3 MHz, $CDCl_3$, 298 K, TMS): δ 339.2 ppm. HRMS calculated for $C_{28}H_{34}ClO_6Se$ 593.1204, found 593.1183.

1α-(4-methylophenylselenyl)-17,21-dihydroxy-pregn-4-eno-3,12,20–trioxo-21-acetate (**5e**)

Isolated as a white solid after flash chromatography using petroleum ether/ethyl acetate (8:2); 96% of yield. m.p. (CH_2Cl_2/hexane): 188–190 °C; IR,υ_{max} (cm^{-1}) 2923, 1738, 1663, 1621; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.36 (d, *J* = 7.8 Hz, 2H), 7.06 (d, *J* = 7.8 Hz, 2H), 5.78 (s, 1H), 4.75 (d, *J* = 17.6 Hz, 1H), 4.44 (m, 1H), 2.31 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 1.53 (s, 3H, CH_3), 0.68 (s, 3H, CH_3) ppm; ^{13}C-NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 209.0, 204.7, 197.6, 170.6, 165.3, 138.2, 135.6, 130.0, 124.8, 124.7, 89.0, 67.8, 60.2, 51.7, 51.3, 49.6, 49.5, 42.7, 40.6, 36.8, 34.9, 32.0, 31.6, 23.2, 21.1, 20.5, 18.4, 15.4 ppm; ^{77}Se NMR (76.3 MHz, $CDCl_3$, 298 K, TMS): δ 333.6 ppm. HRMS calculated for $C_{30}H_{37}O_6Se$ 573.1750, found 573.1731.

1α-phenyltio-17,21-dihydroxy-pregn-4-eno-3,12,20-trioxo-21-acetate (**5f**)

Isolated as a white solid after flash chromatography, using petroleum ether/ethyl acetate (8:2); 33% of yield. m.p. (CH_2Cl_2/hexane): 220–221 °C; IR,υ_{max} (cm^{-1}): 1744, 1695, 1613, 1221; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.36–7.26 (m, 5H), 5.80 (s, 1H, C*H*=C), 5.11 (d, 1H, *J* = 17.6 Hz), 4.74 (d, 1H, *J* = 17.6 Hz), 4.43 (t, 1H, *J* = 2.8 Hz), 3.33 (br s, 1H), 3.03–2.98 (m, 2H), 2.87–2.78 (m, 2H), 2.17 (s, 3H, CH_3), 1.55 (s, 3H, CH_3), 0.67 (s, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 209.4, 204.8, 197.5, 170.8, 164.8, 133.8, 133.6, 129.2, 127.9, 124.9, 89.11, 67.9, 57.9, 54.6, 51.4, 49.7, 49.5, 42.4, 39.6, 36.7, 35.1, 32.2, 31.6, 23.3, 20.6, 19.2, 15.5 ppm. HRMS calculated for $C_{29}H_{35}O_6S$ 511.2149, found 511.2169.

1α-benzyltio-17,21-dihydroxy-pregn-4-eno-3,12,20-trioxo-21-acetate (**5g**)

Isolated as a white solid after flash chromatography, using petroleum ether/ethyl acetate (8:2); 11% of yield. m.p. (CH_2Cl_2/hexane): 192–194 °C; IR,υ_{max} (cm^{-1}): 2920, 2845, 1695, 1650; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.32–7.22 (m, 5H, C*H*-Ar), 5.74 (s, 1H, C*H*=C), 5.14 (d, 1H, *J* = 17.5 Hz), 4.71 (d, 1H, *J* = 17.5 Hz), 3.96 (t, 1H, *J* = 2 Hz), 3.65 (d, 1H, *J* = 12.9 Hz), 3.54 (d, 1H, *J* = 12.9 Hz), 2.19 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 0.65 (s, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 209.1, 204.5, 197.1, 170.7, 165.2,

137.4, 129.0, 128.6, 127.2, 124.9, 89.1, 67.6, 57.5, 49.6, 49.3, 42.4, 40.5, 36.9, 36.6, 35.2, 32.2, 23.3, 20.6, 19.3, 15.5 ppm. HRMS calculated for $C_{30}H_{37}O_6S$ 525.2305, found 525.2329.

1α-(4-methylophenyltio-17,21-dihydroxy-pregn-4-eno-3,12,20-trioxo-21-acetate (**5h**)

Isolated as a white solid after flash chromatography, using petroleum ether/ethyl acetate (8:2); 33% of yield. m.p. (CH_2Cl_2/hexane): 193–195 °C; IR,υ_{max} (cm^{-1}) 2943, 1748, 1658, 1217; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ 7.23 (d, 2H, *J* = 8.0 Hz), 7.07 (d, 2H, *J* = 8.0 Hz), 5.79 (s, 1H, C*H*=C), 5.11 (d, 1H, *J* = 17.6 Hz), 4.74 (d, 1H, *J* = 17.6 Hz), 4.33 (t, 1H, *J* = 3.2 Hz, C*H*-S), 3.33 (s, 1H), 3.04 (d, 1H, *J* = 12.6 Hz), 2.99 (d, 1H, 11.2 Hz), 2.83–2.78 (m, 2H), 2.30 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 1.53 (s, 3H, CH_3), 0.68 (s, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz, 298 K, TMS): δ 209.4, 204.8, 197.6, 170.7, 164.7, 138.2, 134.2, 129.9, 124.9, 89.1, 67.9, 57.9, 54.8, 51.4, 49.7, 49.5, 42.4, 39.5, 36.7, 35.1, 32.2, 31.6, 23.3, 21.2, 20.6, 19.2, 15.5 ppm. HRMS calculated for $C_{30}H_{37}O_6S$ 525.2305, found 525.2328.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23063022/s1, Spectroscopic data of all the synthetized compounds.

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Metal-promoted synthesis of steroidal ethynyl selenides having anticancer activity

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ABSTRACT

In this study, we have described simple and efficient methodology for the metal-promoted (Cu_2I_2) preparation of steroidal ethynyl selenides. The compounds were characterized using ^{1}H, ^{13}C and ^{77}Se NMR, FT IR spectroscopy, and MS analysis. A proposed mechanism of the metal-promoted reaction involves the formation of a σ-bound copper acetylide. Due to the fact that organoselenium-based compounds possess a pleiotropic properties and associated with their promising biological activities, in the next step of the study biocompatibility and anticancer activity of the synthesized compounds was evaluated. Steroidal selenides were tested in vitro against estrogen-depend breast cancer cells MCF-7 using spectrophotometric, fluorometric and luminometric methods. Designed selenides showed high hemocompatibility, lack of toxicity against cardiomyocytes cell and great anti-cancer activity in vitro against estrogen-depend breast cancer cells upon 24 h of treatment. We revealed that selenides decrease the viability and proliferation ability of MCF-7 cells by induction of cell apoptosis. It has been noted that the overproduction of reactive oxygen species (ROS) and associated with its activation of Caspase 3/7 are a major mechanism that is responsible of selenides-caused cell death. These data indicate that organoselenium based compounds have great antineoplastic potential and might be developed as novel class of agents dedicated to the breast-cancer therapies.

1. Introduction

In recent years there has been a rapid increase in interest in the synthesis of Se-containing substances due to their pleiotropic properties and relationship to their promising biological activity [1,2]. To date, one of the synthetic representatives of organoselenium compound is ebselen which mimicking glutathione peroxidase (GPx), are able to take a part in catalysis some vital reactions that protect against oxidative damage. Moreover it is known as a multiple biological properties agents that exhibiting therapeutic and protective potential. However, more in depth studies are needed to explain their mode of action and potential of use.

Many studies have been focused on receiving novel seleno-organic compounds to test their therapeutic potential. For this purpose, various derivatives of selenium compounds based on steroid molecules were obtained. Although selenosteroids are not naturally occurring compounds, however many of them are characterized by significant biological activity, which provides them to be interesting in the terms of medical use. In accordance to above, many studies proved that selenosteroids possess a wide range of activities including: anticancer, antimicrobial, and antioxidants properties [3]. Doubtless, currently published data indicated a huge potential of the selenosteroids with some direction for selenoureas derivatives, however further complex studies both in vitro and in vivo level are needed to introduce of them for medical purposes [4].

In selenosteroids structure, selenium can be present as selenide or as a part of a functional group, such as N-arylselenourea, ebselen analogues, isoselenocyanate, etc. (Fig. 1). Steroidal alkynyl selenide (SAS) is an example of a selenosteroid in which selenium is not directly bound to the steroid molecule (Fig. 1c).

Several methods have been developed for the synthesis of alkynyl selenides (AS). New procedures of carbon-selenium bond formation are necessary for general organic synthesis as well as in the pharmaceutical industry. The classical method to receive alkenyl selenide involves the use of BuLi with phenylacetylene followed by the reaction of the formed

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intermediate with phenylselenium bromide (Scheme 1) [5,6].

Methods for AS synthesis using elemental selenium with organomagnesium or lithium compounds were developed in 1990 [7]. Protocols with transition-metal catalysis for AS preparation was introduced by A. L. Braga et al.in 1993 [8,9]. In these procedures, the reaction was promoted by excess or stoichiometric amount of CuI. Catalytic amounts of copper(I) iodide was used in the alternative alkynyl selenides preparation method [10]. AS synthesis was also achieved using copper (II)/tin(II) reagent system and by indium(III) catalysis from diorganodiselenides [11,12]. Synthesis of alkynyl selenides was also performed from terminal alkynes with iodobenzene acetate and Ph_2Se_2 [13], as well as a product of α-acyl-α-(arylseleno)-phosphoranes pyrolysis [14]. Recently a protocol for obtaining alkyl selenides using a binary a benzoyl peroxide/ Ph_2Se_2 system has been presented [15].

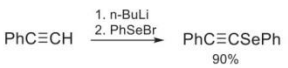
This study originated from our continuing work connected to the synthesis of biological active selenosteroids. Recently, we achieved steroidal β-hydroxy-phenylselenides using Santi's reagent (PhSeZnCl) [16]. The obtained compounds were tested for their antibacterial properties against *Pseudomonas aeruginosa* Xen 5 strain, showing promising bactericidal activity associated with the prevention of biofilm formation. In this context, we accomplished several new steroidal alkynyl selenides. For this purpose, an original procedure was developed for the SAS synthesis via metal-assisted catalysis (Scheme 2).

The second aim of present work is to investigate the biocompatibility and anticancer activity of the synthesized compounds, including elucidation of their mode of action. Selenosteroids were tested in vitro against estrogen-depend breast cancer cells MCF-7 using spectrophotometric, fluorometric and luminometric methods. Biocompatibility of such agents including impact on RBC cells and cardiomyocyte cells was tested as well. In accordance to our knowledge, none of proposed issues were presented to date, which emphasize the novelty of the research presented.

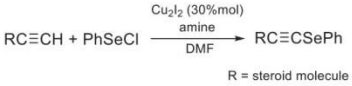
2. Results and discussion

2.1. Chemistry

In this work, we investigated the use of a copper catalyst to functionalize various steroid derivatives having a triple terminal bond to obtain novel selenosteroids as prototypes for testing their biological activity. It is worth mentioning that steroids are very demanding molecules because of their specific structure, rigidity, and reactive functional groups presence. For our initial studies, we chose 3β-acetate-12β-ethynyl-25*R*-5α-spirostane-3β,12α-diol (**1**) as a model substrate. To determine the best conditions for the procedure we examined several reaction parameters (Scheme 3). The effect of solvent and temperature



Scheme 1. Synthesis of phenyl(phenylseleno)acetylene.



Scheme 2. The general formula for steroidal alkynyl selenide synthesis.

(T) was first investigated. Details are provided in Table 1.

Compound **2** was fully characterized by NMR, IR and MS spectroscopies. The presence of senylphelenyl group in the molecule **2** was elucidated from its ^{1}H and ^{77}Se NMR spectrum. The signal of acetylenic proton evanesced (*δ*: 2,52 ppm) and signals from aromatic protons (Se-Ph) appeared (*δ*: 7,53 and 7,31 ppm). In ^{77}Se NMR spectrum, the signal at 267,20 ppm is observed.

By analyzing Table 1, it can be seen that both, the type of reaction medium and the temperature had a significant effect on the course of the steroid selenide formation. It can be observed that desired selenide **2** was not obtained in nonpolar solvents (DCM, toluene) at room temperature and in reflux. In addition to the SAS **1**, the use of THF in reflux gave as a byproduct dimer **3**. The best results were obtained using polar solvent DMF in 100 °C. It is worth mentioning that the conditions of the procedure resulted compatible with the sensitive spiroacetal system [17]. Then we examine the type of copper catalyst. In terms of copper compounds used as catalysts, CuCl, CuOAc, $CuSO_4$, CuO, and Cu_2I_2 were compared, with Cu_2I_2 proving to be the best and only catalyst for the preparation of SAS **2**.

After determination of the catalytic system, the protocol was applied in the reaction of a range of steroidal terminal alkynes **1**, **4**, **6**, and **8**. The results are collected in Table 2.

According to the above-described results, we propose the mechanism of the metal promoted SAS synthesis. Originally, copper(I) intermediate **I** is formed from Cu_2I_2 and steroidal alkyne **1**. Then, σ-bound copper acetylide **II** created in the presence of amine reacted with phenylselenyl chloride to obtain desired steroidal alkynyl selenide **2**. Finally, Cu(I) salt is regenerated to complete the cycle. A catalytic cycle involving the formation of a copper acetylide for a steroidal selenide obtaining reaction is presented on Scheme 4.

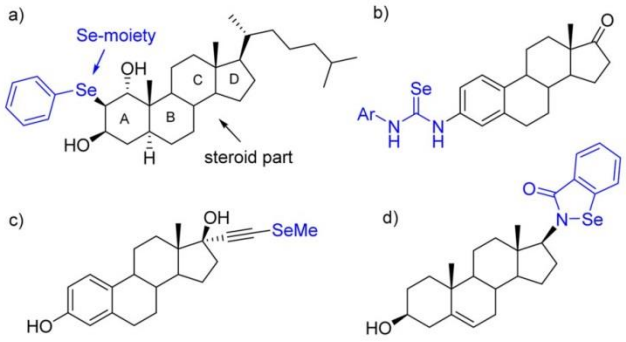
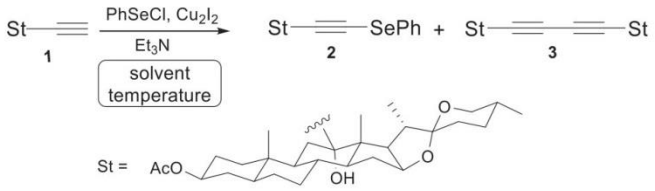


Fig. 1. Examples of selenosteroids: a) selenide - selenium is bonded directly to the steroid molecule, b) - d) selenium is present in a group bonded to the steroid.



Scheme 3. Synthesis of steroidal alkynyl selenide **2**.

Table 1
Investigation of the solvent and temperature reaction impact. Abbreviations: Et_3N = trietylamine, DCM = dichloromethane, THF = tetrahydrofuran, DMF = dimethylformamide, RT = room temperature, R = reflux.

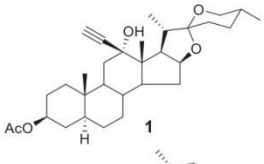
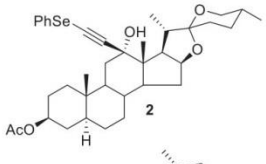
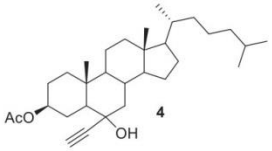
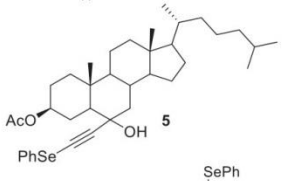
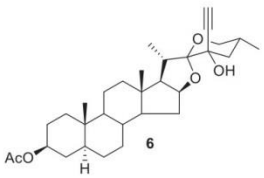
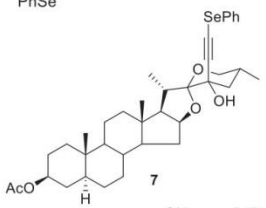
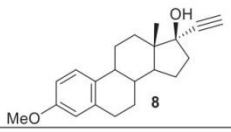
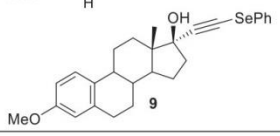
Entry	Solvent	T	Yield 2	Yield 3
1	DCM	RT	–	–
2	DCM	R	–	–
3	THF	RT	–	–
4	Toluen	RT	–	–
5	Toluen	R	–	–
6	DMF	RT	–	–
7	THF	R	16%	25%
8	DMF	100 °C	83%	–

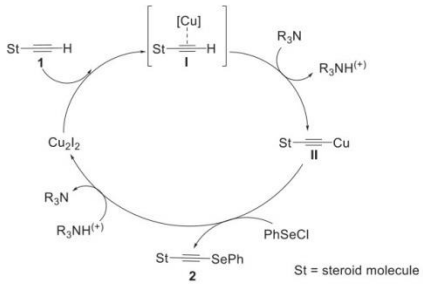
2.2. Biological activity

Based on statistical data presented by WHO, it is established that in 2020, more than 2.3 million women have been diagnosed with breast cancer and there were 680,000 deaths globally. Due to the fact that on the end of 2020, there were about 8 million women alive in which breast cancer has been detected in the past 5 years, caused it the world's most prevalent neoplastic diseases [18,19].

An important limitation in the clinical introduction of novel class of anticancer agents which additionally possessed a membrane-destruction activity is their low selectivity and in effect the potential damage to host cells membranes such as RBC cells [20]. To evaluate the ability of tested compounds **2**, **5**, **7**, and **9** to induce toxic effects against representatives of host cells, a hemolysis assay was performed. The above mentioned assay is crucial because there is no standard preclinical in vivo examination method to perform complex evaluation the hemolytic reaction of therapeutic agent. Additionally, it should be emphasized that hemolytic

Table 2
The scope of reaction.

Entry	Substrate	Product	Yield
1.			83%
2.			64%
3.			96%
4.			63%



Scheme 4. The plausible mechanism of reaction.

anemia (HA) is an important hematological problem which is associated with the treatment by antineoplastic agents such as anthracycline derivatives and the occurrence of HA during anticancer treatment was > 50% [21].

In the performed experiment, isolated from human red blood cells have been used as a model of host membranes, providing for the assessment of membrane-destruction properties of obtained compounds **2**, **5**, **7**, and **9**. As presented in Fig. 2A the synthesized products are characterized by low hemolytic activity when added in the higher dose – 100 μg/mL. All tested compounds caused hemolysis between 1% and 2.5% which is below the international standard level of 10% which is proposed for pharmaceuticals agents [22,23]. Thus, based on obtained results synthesized steroidal selenides **2**, **5**, **7**, and **9** have been confirmed to have good hemocompatibility and will be suitable to be used for intravenous administration.

Statistical significance for the selenides vs. *control was marked with (*); Concentrations dependent response of selenides marked with ($) Comparison to compound 2 marked with (^); Comparison to compound 5 marked with (&),comparison to compound 7 marked with (#), $p \leq 0.05$. The data presented constitute average results from three measurements ± SD and was normalized to control.*

One of major undesired outcome of chemotherapy is negative impact on cardiac structure and function. It is established that in breast cancer patients - cardiac-related mortality are strongly associated with therapy by anthracyclines, as well as by hormonal therapy, tyrosine kinase inhibitors, anti-VEGF drugs. The aforementioned treatment might lead to cardiac-ischemia, vascular thromboembolism and other syndromes of heart failure [24]. To evaluate the cardiotoxicity of synthesized selenides, H9C2(2−1) cells, which is known as a s a model cells for in vitro studies, has been engagement [25]. Results presented in Fig. 2B indicated that tested agents at all used concentrations did not significantly decrease the viability of cardiomyocytes if compared to control. In turn, evaluation of proliferation and metabolic activity of treated cells indicated that selenide 9 at concentration 100 $\mu g \cdot mL^{-1}$ causes statistically marked inhibition of cell proliferation ~ 70% (Fig. 2C).

To determine the inhibitory effects of designed agents, breast cancer cells were treated with selenides **2**, **5**, **7**, and **9** at concentrations range 0–100 $\mu g \cdot mL^{-1}$ for 24 h followed by investigation of cytotoxicity and the ability to proliferate. As shown in Fig. 2D, tested agents significantly inhibited viability of treated cells when compared to the control group. In the case of the lowest applied concentration (10 $\mu g \cdot mL^{-1}$) results show that besides compound **2**, compounds **5**, **7**, and **9** caused a statistically marked decrease in the percentage of viable cells by around 50%. Further increasing the concentration up to 50 and 100 $\mu g \cdot mL^{-1}$ indicated a cytotoxic effect for all evaluated steroidal selenides. The most depletion of viable cells was noted after treatment of MCF-7 cells by compounds **7** and **9**, and viability has been classified below 20%. Important is also the fact that in the case of the aforementioned compounds, reductions in the viability of the cells in a dose-dependent manner have been detected.

The cytotoxic effects of synthesized agents were also analyzed using a proliferation assay (Fig. 2E). Accordingly, the proliferation capability of cells reflected by the ability to conversion of MTT to a water-insoluble colored formazan derivative was spectrophotometrically detected after 24 h incubation. Statistically significant inhibition of the proliferation has been detected after treatment of the cells by 10 $\mu g \cdot mL^{-1}$ – only for selenide **7**, 50 $\mu g \cdot mL^{-1}$ for **5**,**7**,**9**, and 100 $\mu g \cdot mL^{-1}$ for all tested agents. However, it should be emphasized that after incubation of the cells by agent **9** applied at concentrations of 50 and 100 $\mu g \cdot mL^{-1}$ considerable inhibitory effect has been noted. The ability of the cell to divide has been reduced below 20%. In the case of the rest tested agents, the inhibition of cell proliferation has been classified as moderate-strong efficacy and was noted at around 50%.

To better indicate the potential of synthesized agents and compare them to currently used chemotherapeutic agents, the IC50 value of tested compounds and doxorubicin (DOX) - a widely used drug in the treatment of breast cancer, has been calculated. Results are presented in Table 3.

To examine whether synthesized selenides **2**, **5**, **7**, and **9** disrupt redox balance in treated cancer cells, oxidative stress-associated parameters including ROS was investigated. For this purpose luminometric based assay has been used [26]. As demonstrated in Fig. 2F, treatment of breast cancer cells with tested products, especially in the case of selenide **9** leads to a significant increase in ROS production. After normalization to untreated control, the number of ROS increased 10-fold when 100 $\mu g \cdot mL^{-1}$ of agent **9** was applied.

To more precisely explore the molecular mechanism involved in the observed activity, we investigated whether the caspase 3/7 signaling pathway might be engaged in this process. As shown in Fig. 2G in the MCF-7 cells, the maximum caspase 3/7 activity is noted after treatment by compound **9**. Taken together, obtained data indicate that caspase-depended mechanism associated with ROS generation is responsible for cell death.

It is established that to overcome the problems associated with ineffective cancer treatment and to achieve higher compatibility with the host cells, numerous studies including introducing new structural elements such as heteroatoms, functional groups, or the use of drug carriers have been described [27–29]. In view of the above, the potential application of organoselenium derivatives of steroid compounds such as selenosteroids has been explored [3]. To date, it is indicated that this class of compounds exerts a wide range of biological activities such as anticancer, anti-inflammatory, and antimicrobial properties. Previous reports performed by Fuentes-Aguilar et al., it was indicated that anticancer activity is strongly dependent on the nature of the organoselenium group, its position on the steroid, and the substituents on the N-aryl fragments. Authors indicated that in the case of breast cancer cells the potential mode of action is associated with the cells' cycle arrest and treatment by tested compounds caused accumulation of cells in the G1 phase of the cell cycle [4]. In another report published by Cui *et.al.*, the family of selenadiazolylpregnenolone derivatives was synthesized and their antineoplastic potential has been tested against different cells line including the representative human breast cancer cells T47D and MCF-7 cell lines. Interesting was the fact that besides the above-mentioned kind of cells (T47D and MCF-7), synthesized agents exert high antiproliferative activity. In effect lack of anticancer efficacy, IC50 > 100 μM, against MCF-7 and T47D breast cancer cell lines has been observed [30]. In the recently published study, varied in vitro antiproliferative activities against MCF-7 cells have been detected. Authors showed that 17-selenocyanopregnenolone and their derivatives are able to inhibit proliferation of T47D and MCF-7 cells. In turn, 21-selenocyanopregnenolone and their derivatives showed a lack of cytotoxicity to all tested cell lines [31]. The structure-activity relationship has been also illustrated in report published by Huang et al. For example, authors indicated that in the case of 3-(8-selenocyanooctyloxy)-estradiol the IC50 value is < 10 μM, while in the case of

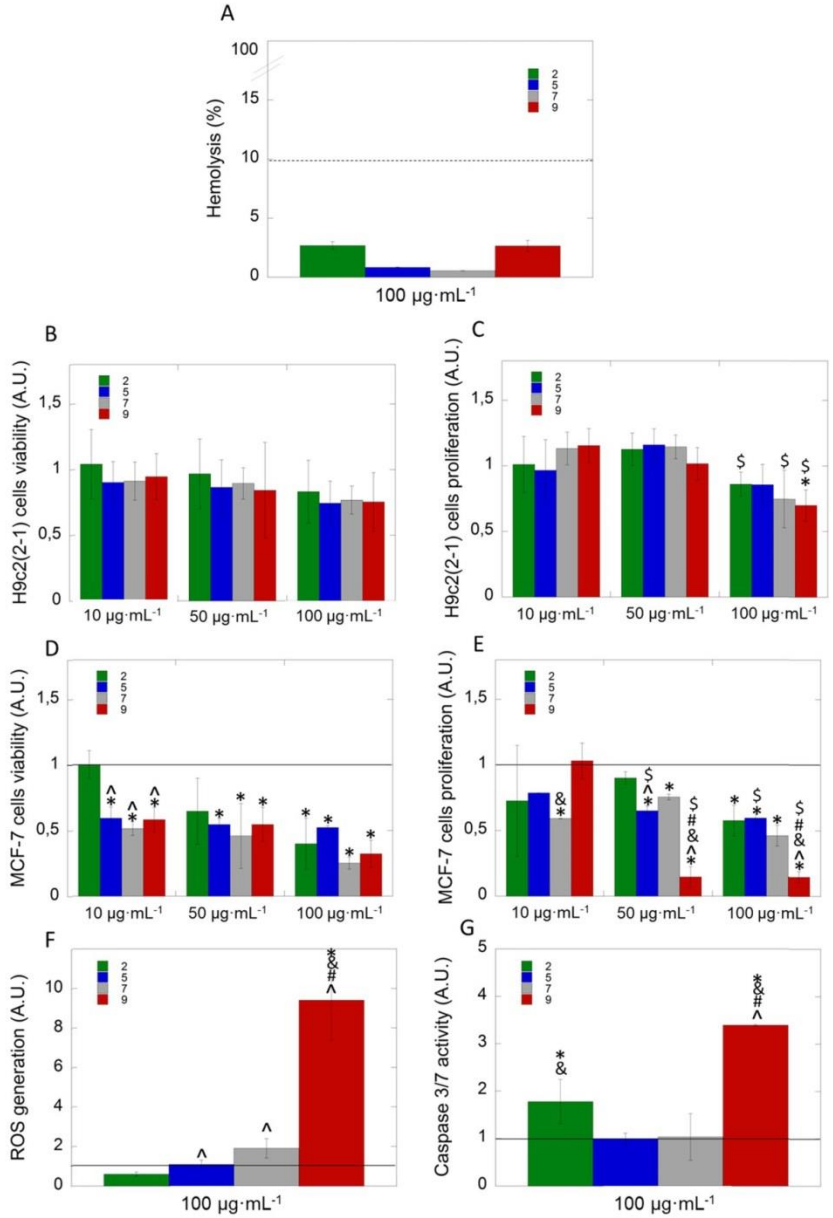


Fig. 2. Biocompatybility and anticancer activity of selenides **2**, **5**, **7**, and **9**. Lack of hemolytic activity (A). Viability (B) and proliferation (C) of cardiomyocyte cells after treatment by selenides. Depletion of MCF-7 cell viability after treatment by selenides (D) and antiproliferative activity of selenides (E). Generation of reactive oxygen species (F) and increasing activity of caspase 3/7 (G) after treatment of MCF-7 cells.

Table 3
IC50 value of tested agents in comparison to DOX.

Compound	IC 50 [μM]	
	Estrogen-depend breast cancer MCF-7 cells	Cardiomyocyte H9c2 (2–1)
2	> 100	> 100
5	> 100	> 100
7	54 ± 18	> 100
9	77 ± 17	> 100
DOX	49 ± 2,35	5,5 ± 1,90

3-Methoxy-17-(o-selenocyanomethylbenzoyloxy)estradiol the 8-fold higher IC50 value > 80 μM has been noted [32].

3. Experimental section

3.1. Synthesis

3.1.1. General information

Reactions were conducted in round-bottom flasks and were stirred with Teflon coated magnetic stirring bars. Flash chromatography was performed with silica gel, pore size 40 A (70–230 mesh) unless otherwise stated. All reactions were monitored by TLC on silica gel plates 60 F254. NMR experiments were performed in a Bruker Advance 400 spectrometer. ^{1}H and ^{13}C NMR chemical shifts (δ) are reported in parts per million (ppm), and they are relative to TMS (0.0 ppm), and the residual solvent peak ($CDCl_3$, 7.27 for ^{1}H NMR, and 77.0 ppm for ^{13}C NMR). ^{77}Se chemical shifts (δ) are reported in parts per million (ppm), and they are relative to diphenyl diselenide (464 ppm) in $CDCl_3$. Data are reported as follows: chemical shift (multiplicity, coupling constants, where applicable, and the number of hydrogen atoms). Abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublet), m (multiplet). Coupling constant (J) is quoted in Hz to the nearest 0.1 Hz. High-resolution mass spectrometry (HRMS) measurements were performed using a Agilent 6520 QTOF instrument. IR spectra were obtained in a $CHCl_3$ solution with an Nicolet 6700 with Smart Orbit pickup, and data are reported in cm^{-1}. Melting points were determined by a Kofler bench (Boetius type).

3.1.2. General procedure

To a solution of alkyne steroidal derivative 1 equiv. in anhydrous DMF was added 1.1 equiv. of PhSeCl, 30% mol CuI and a few drops of Et_3N. Resulted mixture was refluxed for 2 h. Then, the reaction mixture was poured into water and extracted with DCM (3×15ml). Combined organic layer was dried with Na_2SO_4, and the solvent was removed under vacuum. The products were purified by liquid chromatography.

3α-acetyl-12β-phenylselenaethynyl-12α-hydroxyhecogenin (2) was isolated as a white solid with 83% of yield after column chromatography; eluent hexane/ethyl acetate (9:1) Melting point 209–211°C (CH_2Cl_2/hexane); ^{1}H NMR (400 MHz, CDCl3) δ/ppm: 7.53 (m, 2 H), 7.31 (m, 3 H), 4.68 (m, 1 H), 4.40 (m, 1 H), 3.46 (dd, $J = 3,3$ $J = 9,8$ Hz, 1 H), 3.36 (m, 1 H), 2.40 (q, $J = 6,2$, $J = 8.5$ Hz, 1 H), 2.03 (s, 3 H), 1.03 (s, 3 H), 0,95 (d, $J = 6.9$ Hz, 3 H), 0.86 (s, 3 H), 0.79 (d, $J = 6.3$ Hz, 3 H); ^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 170.6 (C), 129.9 (CH x 2), 129.5 (CH x 2), 128.1 (C), 127.4 (CH), 109.2 (C), 106.8 (C), 80.0 (CH), 73.5 (CH), 72.8 (C), 66.8 (CH_2), 64.4 (C), 53.8 (CH), 48.4 (CH), 48.3 (C), 48.1 (CH), 44.5 (CH), 42.1 (CH), 36.5 (CH_2), 35.8 (CH_2), 35.2 (C), 34.8 (CH), 33.9 (CH_2), 31.6 (CH_2), 31.5 (CH_2), 31.4 (CH_2), 30.3 (CH), 28.8 (CH_2), 28.4 (CH_2), 27.3 (CH_2), 21.4 (CH_3), 17.1 (CH_3), 15.7 (CH_3), 14.2 (CH_3), 12.1 (CH_3); ^{77}Se NMR (76.3 MHz, $CDCl_3$) δ/ppm: 267,20; IR ($CHCl_3$) υ/cm-1: 3461, 2925, 1733, 1712, 1454. HRMS calcd for $C_{37}H_{50}O_5SeH^+$ 655.2902, found 655.2876.

3α-acetyl-6ζ-phenylselenaethynyl-6ζ-hydroxycholestan (5) was isolated as a white solid with 64% of yield after column chromatography; eluent hexane:ethyl acetate (9:1). Melting point 139–141 °C (CH_2Cl_2/hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.50 (m, 2 H), 7.32 (m, 3 H), 4.79 (m, 1 H), 2,30 (m, 1 H), 2.05 (s, 3 H), 1.04 (s, 3 H), 0.91 (d, $J = 6.5$ Hz, 3 H), 0.88 (d, $J = 6,7$ Hz, 3 H), 0,86 (d, $J = 6,6$ Hz, 3 H), 0.70 (s, 3 H); ^{13}C NMR (400 MHz, CDCl3) δ/ppm: 170.4 (C), 129.5 (CH x 2), 128.9 (CH x 2), 128.6 (C), 127.1 (CH), 107.9 (C), 73.6 (CH), 71.8 (C), 63.3 (C), 56.2 (CH), 55.6 (CH), 53.7 (CH), 50.7 (CH), 46.4 (CH_2), 42.7 (C), 39.8 (CH_2), 39.5 (CH_2), 38.2 (CH_2), 36.1 (CH_2), 36.0 (C), 35.8 (CH), 31.1 (CH), 28.4 (CH_2), 28.2 (CH_2), 28.0 (CH), 27.3 (CH_2), 24.2 (CH_2), 23.8 (CH_2), 22.8 (CH_3), 22.5 (CH_3), 21.4 (CH_3), 20.9 (CH_2), 18.7 (CH_3), 15.3 (CH_3), 12.1 (CH_3); ^{77}Se NMR (76.3 MHz, $CDCl_3$) δ/ppm: 265,03; IR (CHCl3) υ/cm^{-1}: 3477, 2857, 1706, 1466, 1034. HRMS calcd for $C_{37}H_{54}NaO_3Se+$ 649.3100, found 649.3125.

3α-acetyl-23β-phenylselenaethynyl-23α-hydroxyspirostane (7) was isolated as a white solid with 96% of yield after column chromatography; eluent hexane:ethyl acetate (95:5). Melting point 215–217 °C (CH_2Cl_2/hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 7.55 (m, 2 H), 7.3 (m, 3 H), 4,67 (m, 1 H), 4.42 (m, 1 H), 3.53 (dd, $J = 3,1$ $J = 10,5$ Hz, 1 H), 3.33 (t, $J = 11,2$ Hz, 1 H), 2.03 (s, 1 H), 1,13 (d, $J = 6,6$ Hz, 6 H), 0,85 (bs, 6 H); ^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 170.7 (C), 129.4 (CH x 3), 128.3 (C), 127.1 (CH x 2), 109.9 (C), 103.4 (C), 80.9 (CH), 73.6 (CH), 70.1 (C), 66.0 (CH_2), 65.5 (C), 63.6 (CH), 56.2 (CH), 54.2 (CH), 44.9 (CH_2), 44.6 (CH), 41.3 (C), 39.9 (CH_2), 36.7 (CH_2), 36,3 (CH), 35.6 (C), 35,0 (CH), 34.0 (CH_2), 32.2 (CH_2), 31.9 (CH_2), 29.8 (CH), 28.4 (CH_2), 27.4 (CH_2), 21.4 (CH_3), 20.9 (CH_2), 16.8 (CH_3), 16.5 (CH_3), 16.2 (CH_3), 12.2 (CH_3); ^{77}Se NMR (76.3 MHz, $CDCl_3$) δ/ppm: 271,52; IR ($CHCl_3$) υ/cm^{-1}: 3559, 2844, 1722, 1254, 1037; HRMS calcd for $C_{37}H_{50}O_5Se^+$ 654.2823, found 655.2862.

17α-[(Phenylseleno)ethynyl] – 17β-estradiol (9) was isolated as a white solid with 63% of yield after column chromatography; eluent hexane:ethyl acetate (95:5). Melting point 171–173 C (CH_2Cl_2/hexane); ^{1}H NMR (400 mHz, $CDCl_3$), δ [ppm]: 7.56 (d, J=7.3 Hz, 2 H), 7.32 (m, 2 H), 7.25 (m, 2 H), 6.75 (dd, $J_1 = 2,6$ Hz, $J_2 = 8,5$ Hz, 1 H), 6.67 (d, $J =$ 2,5 Hz, 1 H), 3,80 (s, 3 H), 2.89 (m 2 H), 0.94 (s 3 H); ^{13}C NMR (400 MHz, $CDCl_3$) δ/ppm: 157,36 (C), 137,36 (C), 132.5 (C), 129.4 (CH x 2), 128.9 (CH), 128.8 (CH), 127.0 (CH), 126.3 (CH), 113.7 (CH), 111.4 (CH), 107.3 (C), 81,1 (C x 2), 65.5 (C), 55.1 (CH_3), 49.7 (CH), 47.7 (C), 43.5 (CH), 39.4 (CH), 39.2(CH_2), 33.0 (CH_2), 29.8 (CH_2), 27.2 (CH_2), 26.4 (CH_2), 22.9 (CH_2), 12.8 (CH_3); IR ($CHCl_3$) υ/cm^{-1}: 3419, 2927, 1608, 1576, 1498, 1062; ^{77}Se NMR (76.3 MHz, $CDCl_3$) δ/ppm: 267,75; HRMS calcd for $C_{27}H_{30}NaO_2Se^+$ 489,1309 found 489.1314.

3.2. Biological activity

3.2.1. Hemocompatibility assessment

The hemocompatibility of the tested agents was examined using hemolysis assay. First, the fresh human red blood cells (RBCs) subjected from healthy volunteers were suspended in phosphate-buffered saline (PBS) to establish hematocrit ~5%. Then, tested compounds were added at the concentration 100 μg·mL^{-1} and incubated for 1 h at 37 °C. Next, after centrifugation, the relative hemoglobin concentration in supernatants was spectrophotometrically measured at wavelength 540 nm. The 0% hemolysis was taken from samples after the addition of 10 μL PBS, while the 100% hemolysis was taken from samples in which 1% Triton X-100 was added to disrupt all cell membranes.

The hemolytic activity of the tested agents was evaluated in blood samples from adult healthy adult volunteers under IRB approval: R-I-002/254/2019. This study was approved by the Institutional Review Board (IRB) of The Medical University of Bialystok. All subjects provided informed written consent, and collected samples were anonymous.

3.3. Cytotoxicity studies

3.3.1. Cell culture

Estrogen-dependent MCF-7 human breast cancer cells and cardiomyocyte TheH9c2(2–1) cell line, were obtained from ATCC and maintained in Dulbecco's Modified Eagle Medium (DMEM) with the

addition of 10% heat-inactivated fetal bovine serum and 100 μg·mL^{-1} penicillin/streptomycin at 37 °C in a humified atmosphere containing 5% CO_2. For all experiments, cells were plated in 96 well plates at a seeding density of 10^4/0.32 cm^2 and cultured to reach a confluency of around 75–85%.

3.3.2. Cell viability and proliferation

The viability of cardiomyocyte H9c2(2−1) cells and MCF-7 breast cancer cells were assessed using a Neutral Red test. Briefly, the tested agents was added to cells at the following concentrations:0, 10, 50 and 100 μg·mL^{-1} and left for further incubation for 24 h at 37 °C. Tested agents has been resolved in DMSO and then added in volume from 0.5 up to 1 μL per 100 μL of medium in the well. To exclude the toxic effect of the solvent against untreated control cells, 1 μL of DMSO per well has been added. The viability of cells was carried out by spectrophotometric methods. For this purpose, the neutral red solution (0.33%) was added to each well, and the mixture was incubated for 2–3 h. In the next step, neutral red was removed, and the cells were gently rinsed with Neutral Red Assay Fixative solution. After 10 min the fixative solution was removed, and the incorporated dye was then solubilized in an adequate volume of solubilization solution (100 μL). Finally, the absorbance at a wavelength of 540 nm was measured and normalized of control.

To assess the ability of synthesized agents to inhibit the cell proliferation the MTT- based assay or resazurin-based assay has been performed. In brief, after the addition of the tested agents to cardiomyocyte H9c2(2−1) cells and MCF-7 breast cancer cells at the concentration ranging 0 10, 50 and 100 μg·mL^{-1} and 24-hours incubation, the MTT solution at a final concentration of 0.5 mg·mL^{-1}or 10 μL of resazurin was added to each well, followed by further incubation for 3 h. The absorbance of samples was measured using microplates reader at 570 nm. In the case of MTT-assay, before plate reading the unsolvable formazan has been resolved in 100 μL of dimethyl sulfoxide (DMSO) and carefully shaking. The absorbance value obtained in cultures of control cells (without tested agents) was taken as 100% and then results were normalized of control.

3.3.3. Detection of reactive oxygen species

Detection of ROS in the treated cell by tested agents applied at concentration 100 μg·mL^{-1} was based on the chemiluminescence of luminol. The generation of ROS was investigated by adding 100 μL of luminol solution (5 mM in phosphate-buffered saline) to cultured cells. The chemiluminescence was measured using Varioscan Lux microplates reader. The mean luminescence of the untreated cells served as the reference for results normalization to control.

3.3.4. Caspase 3/7 activity

Caspase 3/7 activity after treatment of MCF-7 cell with created selenides at concentration 100 μg·mL^{-1} was estimated using the luminometric assay. In brief, after 24 h incubation the plates containing treated cells was removed from the incubator and allow plates to equilibrate to room temperature. Then, 100 μL of Caspase-Glo® 3/7 Reagent was added to each well and gently mixed using a plate shaker at 300–500 rpm for 30 s. After that the plate has been incubated at room temperature for 1 h. Finally, the luminescence of each sample was measure in a plate-reading luminometer. The mean luminescence of the untreated cells served as the reference for results normalization to control.

3.4. Statistical analysis

Statistical analyses were performed using Statistica 13.3 software (StatSoft Inc., Tulsa, OK, USA). The data were analyzed using standard statistical analyses, including Student's' t Test (for independent samples). p-values less than 0.05 were considered significant. The IC50 values were calculated using nonlinear regression (three parameters) in GraphPad Prism.

4. Conclusion

In this study, we have prepared and examined the steroidal ethynylselenides to further our understanding of the role of the structure of selenosteroids in the antiproliferative and antioxidant activity. Using metal-promoted methodology (Cu_2I_2 in the presence of Et_3N) we synthesized selenosteroids **2**, **5**, **7**, and **9** with very good yield. Selenosteroids are probably formed by a catalytic cycle in which σ-bound copper acetylide is the crucial intermediate. This is the first report of Cu salt as a catalyst for steroidal ethynylselenides obtaining.

Nowadays, various therapies are proposed as an option for treating breast cancer. However, high numbers of drug-resistant tumors and significant side effects of currently used antineoplastics markedly impede the medication process. For this purpose, the evaluation of hemocompatibility properties as well as their impact on viability on representatives of healthy cells such as cardiomyocyte of the potential chemotherapeutic agents that are dedicated for intravenous injection is the most important parameter to ensure its good compatibility with the circulatory system. Our data have shown that all proposed steroidal selenides were characterized by high rate of hemocompatibility and lack of in vitro cardiotoxicity. Results from cytotoxicity studies indicated that tested agents are able to decrease viability and inhibit the proliferation of treated cells in a dose-depend manner. The decrease in cells survival below 30% and decline in proliferation up to 20% have been obtained for 17α-[(phenylseleno)ethynyl]− 17β-estradiol (**9**). Another new finding according to the obtained results is that both ROS generation and caspase 3/7 activity are very much affected by compound **9**. Incubation with selenide **9** increases ROS generation and caspase 3/7 activity 9 and 3.5 fold respectively.

CRediT authorship contribution statement

Pawel A. Grzes: Investigation. **Agata Sawicka:** Investigation. **Katarzyna Niemirowicz-Laskowska:** Investigation. **Przemysław Wielgat:** Investigation. **Diana Sawicka:** Investigation. **Halina Car:** Supervision, Funding acquisition. **Izabella Jastrzebska:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2022.106232.

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Białystok, 15.06.2023

OŚWIADCZENIE

Oświadczam, że w pracy: I. Jastrzębska, Stefano Mellea, Valerio Salerno, Pawel Adam Grzes, Leszek Siergiejczyk, Katarzyna Niemirowicz-Laskowska, Robert Bucki, Bonifacio Monti, Claudio Santi; pt. PhSeZnCl in the Synthesis of Steroidal β-Hydroxy-Phenylselenides Having Antibacterial Activity, *Int. J. Mol. Sci. 2019, 20, 2121; doi:10.3390/ijms2009212,* mój udział polegał na opracowaniu metodologii syntezy, konceptualizacja, nadzór nad projektem, pisanie manuskryptu oraz pozyskiwanie funduszy.

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Białystok, 23.05.2023

OŚWIADCZENIE

Oświadczam, że w pracy: P.A. Grzes, A. Sawicka, K. Niemirowicz-Laskowska, P. Wielgat, D. Sawicka, H. Car, I. Jastrzebska, Metal-promoted synthesis of steroidal ethynyl selenides having anticancer activity, *Journal of Steroid Biochemistry and Molecular Biology 227*, 106232 (2023) mój udział polegał na części badań dotyczących opracowania wstępnych procedur syntezy selenków steroidowych.

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Perugia, 22th June 2023

To whom it may concern

I declare, that my participation in the work: Grześ, P.A.; Monti, B.; Wawrusiewicz-Kurylonek, N.; Bagnoli, L.; Sancineto, L.; Jastrzebska, I.; Santi, C. Simple Zn-Mediated Seleno- and Thio-Functionalization of Steroids at C-1 Position. *Int. J. Mol.Sci.* ***2022***, *23, 3022*. https://doi.org/10.3390/ijms23063022 is related to the investigation process.

Sincerely

Prof. Luana Bagnoli

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OŚWIADCZENIE

Oświadczam, że w pracy: I. Jastrzebska, P. A. Grzes, K. Niemirowicz-Laskowska, H. Car, Selenosteroids - promising hybrid compounds with pleiotropic biological activity: synthesis and biological aspects, *Journal of Steroid Biochemistry and Molecular Biology* 213, 105975 (2021) mój udział polegał na opisaniu części dotyczącej właściwości biologicznych.

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Leszek Siergiejczyk

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Halina Car