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Design and synthesis of substituted morpholin/piperidin-1-yl-carbamodithioates as promising vaginal microbicides with spermicidal potential ☆

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ABSTRACT

A series of seventeen morpholin/piperidin-1-yl-carbamodithioate (**3**–**19**) were synthesized as topical vaginal microbicidal spermicides. The synthesized compounds were evaluated for their anti-*Trichomonas* activity against MTZ susceptible and resistant strains along with their spermicidal and antifungal potential. All the synthesized compounds were assessed for their safety through cytotoxic assay against human cervical cell line (*HeLa*) and compatibility with vaginal flora, *Lactobacillus*. The study identified eleven dually active compounds with apparent safety. The plausible mode of action of these compounds was through sulfhydryl binding, confirmed via reduction in available free thiols on human sperm. The most promising compound **9** significantly inhibited ($P < 0.001$) thiol-sensitive sperm hexokinase. The stability of compound **9** in simulated vaginal fluid (SVF) was performed via HPLC-PDA method, which supported its utility for vaginal administration.

According to UNAIDS report 2013, currently about 35 million people are living with AIDS/HIV.[1] Seventy-five percent cases of HIV acquisition are through heterosexual contacts and sexually transmitted infections (STIs), attributable to unsafe sexual behavior.[2,3] Each year, an estimated 500 million people acquire at least one of four STIs: chlamydia, gonorrhoea, syphilis and trichomoniasis.[4] *Trichomonas vaginalis* (TV) is exclusively sexually transmitted in adults, accounting for 30% of STI cases[5] and associated with pelvic inflammatory disease (PID), vaginitis and pregnancy complications in women.[6,7] TV infection resulted in impaired vaginal milieu, eventually favoring HIV transmission.[8,9] It is well established that being receptive partners, women are twice vulnerable to HIV infection during coital act than their male partners.[10] However, consistent condom use was found to reduce HIV transmission by 64% and STI transmission by 42%,[11] but its use is not women controlled. Vaginal microbicidal spermicide offers women pre-exposure prophylaxis to prevent HIV/STD along with unwanted pregnancy.[12,13]

Initially, nonoxynol-9 (N-9) had been proposed as a spermicidal agent with microbicidal activity (including anti-HIV activity) but on the contrary it increased susceptibility to HIV in clinical trials due to its surfactant action.[14,15] Thus, there is an urgent universal demand to synthesize novel women controlled non-detergent microbicidal spermicide to simultaneously target sperm and *Trichomonas* (STI) for prophylactic contraception.

In our ongoing efforts[16–20] to develop non-surfactant dually active vaginal microbicidal spermicides, carbodithioate derivatives (i–v, Fig. 1) were reported to interact with sulfhydryl (SH) groups present on spermatozoa[21] and *T. vaginalis*[22], which are crucial for their survival. Targeting free thiols would be an impressive approach to arrest sperm and *Trichomonas* in semen since thiols are considered unavailable in vaginal environment due to low pH.[23] Thus, carbamodithioates have been designed as a molecular prototype targeting sperm and *Trichomonas* through a single chemical entity for prophylactic contraception (vi, Fig. 1).

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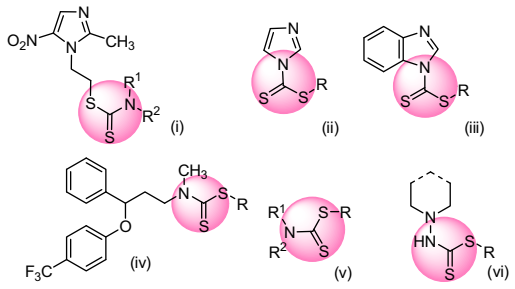


Figure 1. General structures of previously reported carbodithioates (i) metronidazole-carbodithioate (ii) & (iii) azole-carbodithioate (iv) fluoxetine-carbodithioate (v) substituted carbodithioate (vi) general structure of synthesized carbamodithioate derivatives.

Recent SAR studies have revealed that a minor chemical change into the proven structure results in significant potency enhancement.[24–28] Further, it has been also reported that incorporation of one more nitrogen (N) atom into active scaffold has resulted into improved biological activity as exemplified in *N*-amino rhodanine derivatives showing strong insecticidal, antifungal and plant growth inhibitory activity in comparison to rhodanine itself.[29,30] Moreover, *N*-amino piperazine and *N*-amino morpholine have also been reported as biologically active moieties.[31,32] Therefore; it was thought worthwhile adding one more N atom into the established dithiocarbamate structure (**1**) to explore it as vaginal microbicide with spermicidal potential (Fig. 2).

The design and synthesis of carbamodithioates (Fig. 2), their spermicidal, anti-*Trichomonas*, and antifungal activity evaluation, and subsequent safety assessment towards vaginal flora (*Lactobacillus*) and cervical epithelium (*HeLa* cells) have been communicated in this report. The mode of action through SH binding and stability of most promising compound **9** in simulated vaginal fluid (SVF) is also presented.

The substituted morpholin/piperidin-1-yl-carbamodithioate derivatives (**5**–**19**) were synthesized according to the strategy outlined in Scheme 1. The present study reaction was carried out in water without addition of other base to yield substituted morpholin/piperidin-1-yl-carbamodithioate derivatives (**5**–**19**) in 30 min. Significant rate enhancement was observed in water compared to organic solvent. This acceleration is probably due to factors such as hydrophobic effect,[33] enhanced hydrogen bonding in the transition state,[34] etc.

The structures of all newly synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, IR spectroscopy, mass spectrometry (ESMS and HRMS) and elemental analysis. (See Supporting information).

A potent spermicidal activity is a key feature of vaginal microbicides to attract users. The synthesized seventeen compounds (**3**–**19**) were subjected to spermicidal assay involving Sander-Cramer assay[35], where fourteen compounds (except **4**, **12** and **16**) irreversibly immobilized 100% normal human spermatozoa at 0.36–57.08 mM

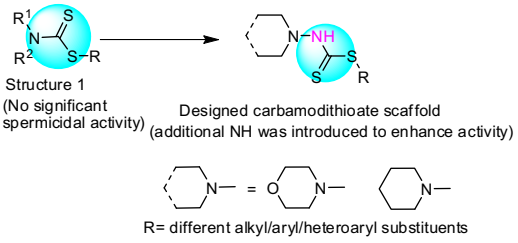
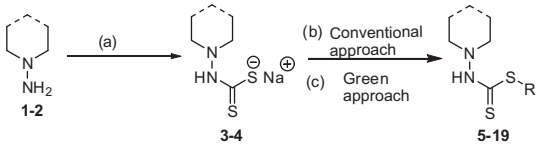


Figure 2. Designing of carbamodithioates.



Scheme 1. (a) CS_2, NaOH, ethyl acetate, 0–5 °C, 3–4 h; (b) alkyl/aryl halide, triethyl amine, methanol, rt, 3–4 h; (c) distilled water, alkyl/aryl halide, 30 min.

concentration (Table 1). Compound **9** and **10** exhibited potent spermicidal activity at 0.36 and 0.48 mM concentrations, respectively, while the marketed drug N-9 had minimum effective concentration (MEC) of 0.8 mM.

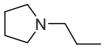
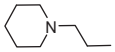
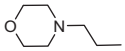
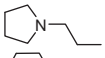
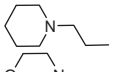
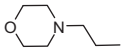
In order to check microbicidal potential, synthesized compounds (**3**–**19**) were further evaluated for anti-*Trichomonas* activity against metronidazole (MTZ) susceptible and resistant TV strains by using standard procedure.[36] Sixteen compounds (except **13**) were found active against MTZ susceptible strain and fifteen (except **13** and **15**) against MTZ resistant strain (Table 1). Remarkable activity was observed in three compounds (**9**, **10** and **19**) at concentration ranging from 0.054–0.056 mM comparable to MTZ (MIC 0.019 mM). Compound **9** (MIC 0.228 mM) was also found as active as MTZ (MIC 0.292 mM) against resistant strain.

The antifungal activity of synthesized compounds (**3**–**19**) was also ascertained against six fungal strains (patient isolates of *Candida albicans*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Trichophyton mentagrophytes*, *Aspergillus fumigates*, and *Candida parapsilosis*-ATCC-220190) and fifteen compounds (except **7** and **15**) were found active at 12.5–50 μg/mL against one or more fungal strains. Surprisingly, fourteen compounds (**3**, **4**, **6**, **8**–**14** and **16**–**19**) were active at 12.5–50 μg/mL against *Trichophyton mentagrophytes* considered as opportunistic sexually transmitted infection.[37] Most potent compound **9** was also active against four fungal strains at 12.5–50 μg/mL, while N-9 was found almost inactive against them.

Structure Activity Relationship (SAR) have been compared by virtue of the spermicidal, anti-*Trichomonas* and antifungal activity results which suggested that piperidine moiety was preferred over morpholine as all piperidin-1-yl-carbamodithioates have shown spermicidal activity ranging from 0.36 to 57.08 mM. Compound **9** and **10** were ~2-fold active than N-9 (MEC, 0.8 mM). The results of spermicidal activity showed that the preference of substituent (R) of piperidin-1-yl-carbamodithioates (**5**–**11**) was ethyl pyrrolidine (**9**) > ethyl piperidine (**10**) > hydroxy ethyl (**7**) > allyl (**6**) > benzyl (**8**) > butyl (**5**) = ethyl morpholine (**11**). Whereas among morpholin-4-yl-carbamodithioates (**12**–**19**), ethyl pyrrolidine (**17**) and ethyl piperidine (**18**) substituents were most desirable.

The anti-*Trichomonas* activity data against MTZ susceptible strain suggested that among sodium salt of carbamodithioates (**3** and **4**), piperidine moiety (**3**, MIC 0.07 mM) was again preferred over morpholine (**4**, MIC 0.64 mM). With piperidine moiety (**5**–**11**), ethyl pyrrolidine (**9**, MIC 0.056 mM) and ethyl piperidine (**10**, MIC 0.054 mM) substituents at sulfur atom possessed notable activity. The order of preferred groups were ethyl pyrrolidine (**9**) > ethyl piperidine (**10**) > ethyl morpholine (**11**) > benzyl (**8**) > allyl (**6**) = hydroxy ethyl (**7**) > butyl (**5**). Morpholine moiety (**12**–**19**) was again found to be less desirable as only compound **19** have shown activity at MIC 0.054 mM while others were active at concentration 0.116–0.268 mM. The activity against MTZ resistant strain was decreased as expected. Among the compounds (**3**–**19**), Sodium morpholin-4-yl-carbamodithioate (**4**) was equipotent for both MTZ susceptible and resistant strain while others lost the activity by 2–8.5-folds as compared to MTZ where activity loss was 15-folds. Piperidino derivatives (**3** and **5**–**11**) were again preferred over morpholino compounds (**4** and **12**–**19**). Compounds

Table 1
Spermicidal and anti-*Trichomonas* activity of the compounds (**3**–**19**) against human spermatozoa and *Trichomonas vagainalis*

Compound	Structure		Spermicidal Activity, MEC in mM[a] (MEC in μg/mL)	Anti-*Trichomonas* activity, MIC in mM[d] (MIC in μg/mL) at 48 h	
		R		Metronidazole susceptible strain	Metronidazole resistant strain
3		Na	50.25 (9.9×10^3)	0.07 (13.86)	0.314 (62.2)
4		Na	—[b]	0.64 (128.0)	0.634 (126.8)
5			49.78 (11.9×10^3)	0.26 (60.58)	0.536 (124.8)
6			12.5 (2.7×10^3)	0.14 (30.38)	1.157 (250.0)
7			2.5 (0.5×10^3)	0.14 (30.80)	0.568 (124.9)
8			21.64 (5.6×10^3)	0.11 (29.37)	0.936 (249.9)
9			0.36 (0.09×10^3)	0.056 (15.34)	0.228 (62.5)
10			0.48 (0.14×10^3)	0.054 (15.55)	0.434 (124.9)
11			49.65 (14.4×10^3)	0.107 (31.03)	0.215 (62.4)
12			—[b]	0.265 (62.28)	0.531 (124.8)
13			49.77(10.8×10^3)	—[c]	—[c]
14			57.08 (13.3×10^3)	0.268 (62.44)	1.07 (249.3)
15			49.77 (11.1×10^3)	0.242 (53.96)	—[c]
16			—[b]	0.116 (31.20)	0.464 (124.8)
17			3.62 (0.9×10^3)	0.226 (62.37)	0.452 (124.7)
18			3.44 (0.9×10^3)	0.215 (62.35)	0.862 (249.9)
19			49.65 (14.4×10^3)	0.054 (15.76)	0.428 (124.9)
MTZ			—[b]	0.019 (3.25)	0.292 (49.9)
N-9			0.8 (0.49×10^3)	ND	ND

[a] Vehicle (control) has 100% motility at the time of testing.
[b] Inactive at 60 mM (15×10^3 μg/mL).
[c] Inactive at 2 mM (250 μg/mL), ND = not done.
[d] The experiments were carried out in triplicate. N-9—Nonoxynol-9, MTZ—Metronidazole.

9 and **11** were as active as MTZ against resistant strains. The results further suggested that dialkylaminoethyl group (**9**–**11** and **17**–**19**) as substituent (R) was more wanted over alkyl/aralkyl groups (**5**–**8** and **12**–**16**) in both piperidino and morpholino moieties.

Substituted morpholin/piperidin-1-yl-carbamodithioates (**3**–**19**) exhibited moderate to good activity against one or more fungal strains (MIC 12.5–50 μg/mL) especially against *Trichophyton mentagrophytes*. The study resulted into eleven compounds (**3**, **5**, **6**, **8**–**11**, **14** and **17**–**19**) with spermicidal, anti-*Trichomonas* (MTZ susceptible and resistant TV strains) and antifungal activity. The most promising compound was 2-(pyrrolidin-1-yl)ethyl piperidin-1-yl-carbamodithioate (**9**) as it showed spermicidal (MEC 0.36 mM), anti-*Trichomonas* against both MTZ susceptible (MIC 0.056 mM) and MTZ resistant TV strains (MIC 0.228 mM) with antifungal activity against four fungal strains (12.5–50 μg/mL).

All the compounds (**3**–**19**) were evaluated for their safety against human cervical (*HeLa*) cell line (Fig. 3a) and compatibility with vaginal flora (*Lactobacillus*) (Fig. 3b) at 200 μg/mL by cell-viability assay with previously reported assay procedures.[19] All the compounds were found highly safe than standard N-9. Compound **9** exhibited safety comparable to control against *HeLa* and *Lactobacillus*. This safety result supports the utility of most active compound **9** as vaginal microbicidal spermicide.

Compound **9** significantly ($P < 0.0001$) reduced the number of free thiols present over the human sperm at spermicidal concentration 0.36 mM (Fig. 4). The decreased number of free thiols suggested that it might be the mechanism of action of compound **9** for spermicidal activity.

After significant reduction of free thiols by compound **9**, it was further hypothesized that hexokinase could be one of the potential targets of –SH binding sperm immobilizing agents. The sperm specific hexokinase, a rate limiting glycolytic enzyme is a thiol-sensitive protein activated by thiol-disulfide inter-conversion after ejaculation.[38] Assay was carried out by using standard procedure.[23] Hexokinase in human sperm exhibited an average specific activity of 8.60 ± 0.20 μmol min^{-1} mg-$protein^{-1}$ that was inhibited significantly to 5.78 μmol min^{-1} mg-$protein^{-1}$ (~32%, $P < 0.001$, Fig. 5) at spermicidal concentration of compound **9** and this could be a key mechanism of its spermicidal action.

SVF (pH 4.2) was considered as a perfect media to determine the stability of molecules that are intended for intra-vaginal use.[39] Stability of most promising compound **9** was performed in SVF

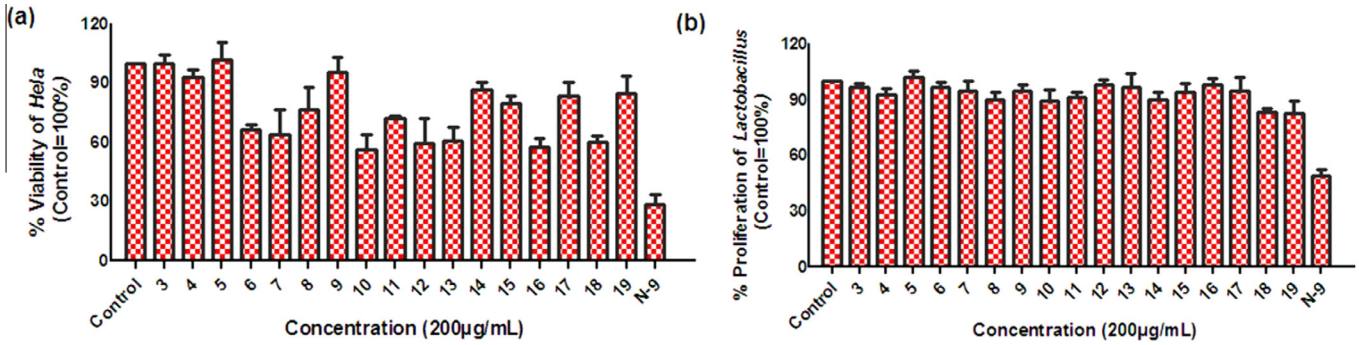


Figure 3. (a) Cytotoxicity of compounds (**3**–**19**) towards Human cervical cell lines (*HeLa*) and (b) Compatibility with normal vaginal flora, *lactobacillus*.

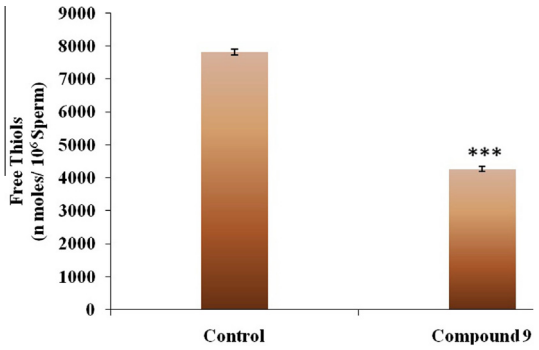


Figure 4. Inhibition of free thiols on human sperm by compound **9** at its spermicidal MEC (0.36 mM). The bars represent means ± SE of three independent experiments using sperm from three different donors. Difference from control was significant ($P < 0.0001$).

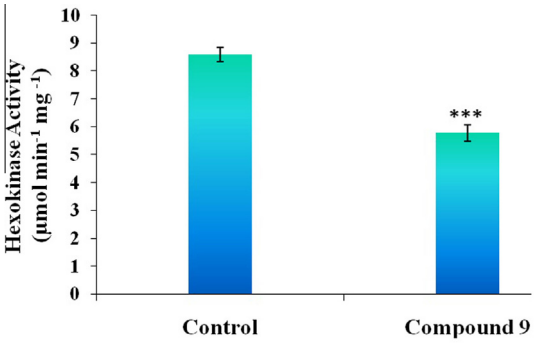


Figure 5. Hexokinase activity of human sperm treated with control and compound **9** (MEC = 0.36 mM) (*** Mean of three independent experiments, $P < 0.001$).

using HPLC-PDA method. The results have shown that the compound **9** was stable in SVF up to 1.0 h (Fig. 6).

Compound **9** was also found stable in pH dependent stability studies in SVF, which could be one of the possible reasons for its potent spermicidal action. This behavior of compound **9** is favorable for its vaginal administration and supports its efficacy for spermicidal activity.

The study has resulted into identification of a novel lead (substituted carbamodithioates) synthesized through a greener approach, which possessed better spermicidal and anti-*Trichomonas* activity profile in comparison to well known marketed spermicide N-9 and FDA approved drug metronidazole. The most promising compound (pyrrolidin-1-yl)ethyl piperidin-1-yl-carbamodithioate (**9**) also possessed antifungal activity against four fungal strains. The extreme safety profile against *HeLa*, compatibility with *lactobacillus* and SVF stability supported its

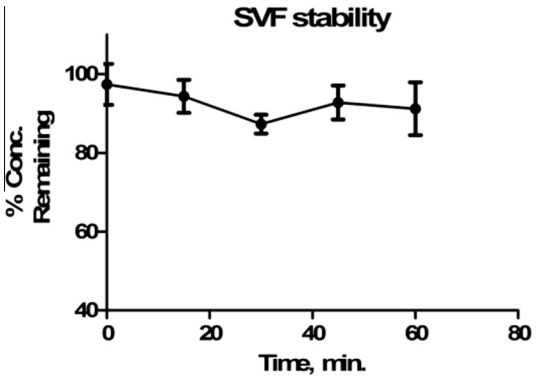


Figure 6. % Conc. remaining vs time plot for SVF stability of compound **9**.

suitability for vaginal application. The mode of action of these compounds was explored via inhibition of free thiol and hexokinase assay. The decrease in number of free thiols and hexokinase activity was suggestive of sulfhydryl binding as the most probable mechanism of action. It may be concluded that introduction of an additional nitrogen atom in alkyl disubstituted aminocarbodithioate moiety resulted into novel carbamodithioates with multiple activities. A further lead optimization may result into drug candidates as vaginal microbicides having spermicidal potential with improved anti-*Trichomonas* activity against MTZ resistant strain, accompanied with enhanced safety to cervico-vaginal cells in comparison with nonoxynol-9.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.10.040.

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