This investigation is continuing despite an elegant total synthesis of (-)-N(8)-norphysostigmine just published by Japanese scientists.[71]

Conclusion

Analogues of colchicine (ethyl carbamate) and thiocolchicine (3-demethylthiocolchicine) which show interesting biological properties have to await further pharmacological and toxicological evaluation to establish their potential clinical usefulness.

The finding, that natural colchicinoids and derived allo congeners bind to tubulin as aS-configurated biaryls, will greatly help in further study of elucidating the mechanism by which they bind to the colchicine binding site on the protein. Systematic efforts to structurally modify compounds of the allo series paid off, since it clearly showed that the methoxy groups at C(1), C(2), and C(9) are required for the binding to tubulin and shifting the acetamido group from C(7) to C(5) afforded an inactive compound.

With efficient synthesis of Calabar alkaloids leading to both optical isomers on hand, it now is up to medicinal chemists to make further molecular changes, which hopefully may lead to clinically useful analgesics and cholinergic agents. Further pharmacological evaluation of (+)-physostigmine and of (-)-N(1)-norphysostigmine, which emerged as interesting compounds, is indicated. In both series of alkaloids discussed, the colchicines and the physostigmines, optical resolution of racemic mixtures and testing optically active isomers instead of racemic mixtures, was pivotal for obtaining useful information.

Acknowledgment. The research presented here could not have been accomplished without the input made by many devoted and hard working scientists. This, in the case of colchicine, included the following Ph.D's: H. G. Capraro, M. A. Iorio, M. Rösner, P. N. Sharma, P. Kerekes, R. Dumont, M. Chrzanowska, A. Muzaffar, Y. Itoh, and O. Boyé from our Laboratory, and C. F. Chignell, E. Hamel, H. I. C. Yeh, J. V. Silverton, G. Clifford, and F. Quinn from intramural collaborations. In the case of physostigmine they were Q. S. Yu, L. A. Chrisey, V. Pabuccuoglu, and M. Brzostowska from our Laboratory, and J. R. Atack, W. X. Albuquerque, and J. L. Flippen-Anderson from extramural collaboration. The interest of B. Witkop and of N. J. Leonard, who spent a year at the NIH as a Fogarty Scholar, in both topics and their many helpful discussions is gratefully acknowledged. I also would like to thank J. Peterson-Mark for editing and typing the manuscript.

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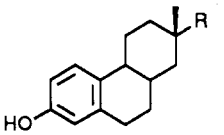
Communications to the Editor

Trifluoromethylacetylenic Alcohols as Affinity Labels: Inactivation of Estradiol Dehydrogenase by a Trifluoromethylacetylenic Secoestradiol

Estradiol dehydrogenase (EC 1.1.1.62) is a pyridine-nucleotide-dependent enzyme that interconverts estradiol and estrone.[1] Because estradiol is more potent than estrone, this enzyme may serve to modulate estrogenic potency in vivo. Also, the role of the enzyme in reproductive endocrinology and in estrogen-dependent neoplasms is of widespread interest.[2-4] Consequently, we have been involved in the development of inhibitors of estradiol dehydrogenase.

We have shown previously that acetylenic secoestradiol **1** is a mechanism-based inactivator of estradiol dehydrogenase.[5] Enzymatic oxidation of **1** (K_m = 79 μM) leads to ketone **2**, a Michael acceptor, which covalently modifies and inactivates the enzyme ($K_i$app = 2.8 μM, limiting $t_{1/2}$ = 12 min).[5-7] Our interest in developing other irreversible inhibitors led us to prepare and evaluate trifluoromethylacetylenic alcohol **3** as an inactivator of estradiol dehydrogenase. We report here that, in contrast to acetylenic alcohol **1** which is a substrate for estradiol dehydrogenase, trifluoromethylacetylenic alcohol **3** is an affinity label. This is the first report in which the (trifluoromethyl)acetylene group has been utlized as an enzyme affinity labeling group.



1: R = (17*S*)-CH(OH)C≡CH
2: R = C(=O)C≡CH
3: R = CH(OH)C≡CCF$_3$
4: R = CHO

Trifluoromethylacetylenic alcohol **3** was synthesized from optically pure secoaldehyde **4**[8] by reaction with lithium (trifluoromethyl)acetylide in 75% yield as a diastereomeric mixture (^{19}F NMR: δ = -50.74, s (broad)).[9] For enzymology, a small sample of the mixture was separated by HPLC, and diastereomers **3a** and **3b** were obtained as noncrystalline solids.[10] The absolute stereochemistry at C-17 in **3a** and **3b** has not been determined.

The diastereomers were evaluated separately as time-dependent inactivators of estradiol dehydrogenase.[11] The

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(9) Satisfactory electron-impact high-resolution mass spectroscopic data as well as NMR (^{1}H, ^{19}F) and IR spectroscopic data have been obtained. ^{19}F NMR values (δ) are reported relative to $CFCl_3$ (δ = 0) as internal standard.
(10) Diastereomers were separated as foams by high-performance liquid chromatography (HPLC) using three tandem Alltech (#60085) 5-μm silica cartridge columns (250 mm × 4.6 mm). Ethyl acetate (15%) in hexanes was used as eluent at a flow rate of 2 mL/min. Retention times for **3a** and **3b** were 25.5 and 26.5 min, respectively.

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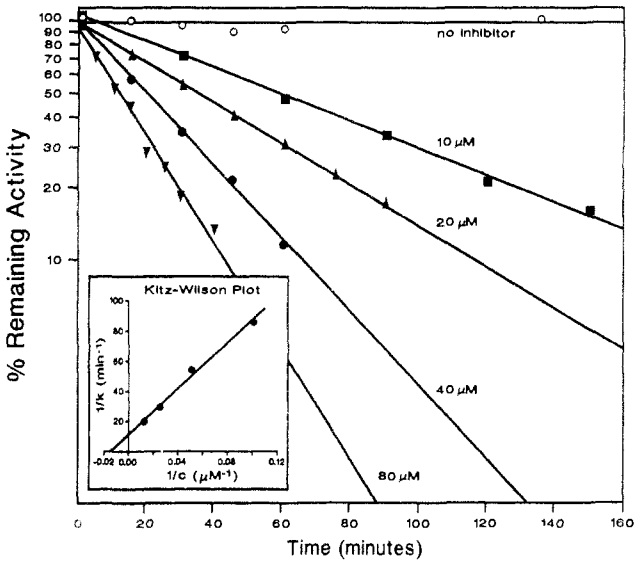


Figure 1. Time-dependent inactivation of estradiol dehydrogenase with different concentrations of trifluoromethylacetylenic alcohol **3b** in the absence of NAD^+.[11] Data points indicate a mean of three experiments (n = 2, for 80 μM) which agreed within 4% at each data point. Inset: Kitz–Wilson double-reciprocal plot.[11] All lines are least-square fits with $r \geq 0.994$.

inactivation profiles for **3a** (data not shown) and **3b** (Figure 1) were similar; both **3a** and **3b** caused a time-dependent inactivation of the enzyme that followed pseudo-first-order kinetics in the *absence* of NAD^+ (i.e. without turnover). Also, no inactivation occurred if glutathione (1 mM) was included in the incubation. These results provided the first indication that, unlike acetylenic alcohol **1**, the trifluoromethylacetylenic alcohols are affinity labels for the enzyme. Since the kinetic parameters are similar for **3a** (K_i = 59 μM, limiting $t_{1/2}$ = 7 min) and **3b** (K_i = 65 μM, limiting $t_{1/2}$ = 8 min), the stereochemistry of the C-17 hydroxyl group appears to be irrelevant for binding and inactivation.

In view of the electrophilic nature of the (trifluoromethyl)acetylene group[12] it appeared that inactivation involved reaction of an enzyme nucleophile with the (trifluoromethyl)acetylene group in **3a** and **3b**. When inactivated enzyme and a control (no inhibitor) were dialyzed for 22 h against buffer (1000× volume), the inactivated enzyme did not recover any activity whereas the control retained 85% of its initial activity. This result is consistent with irreversible inactivation that may be attributed to covalent modification of the enzyme by the (trifluoromethyl)acetylene group.

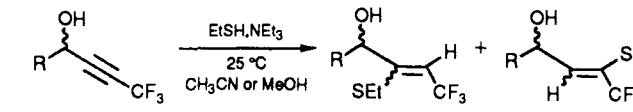
Protection experiments demonstrate that the inactivation is active-site-directed. Thus, when either 17β-estradiol, NAD^+,[13,14] or 17α-estradiol (a competitive inhibitor)[15] were included in the incubations, the rate of inactivation decreased (Table I). Protection by 17α-estradiol reaffirms the irrelevance of the C-17 stereochemistry to the binding process. NADH did not provide any protection, suggesting that inactivation can occur in abortive ternary complexes.

Table I. Summary of Kinetic Parameters from Protection Experiments[a]

substrate/cofactor	k_{app},[b] min^{-1}
none[c]	0.027
17β-estradiol[d]	0.013
17α-estradiol[d]	0.010
NAD^+[e,f]	0.013
NADH[e]	0.025
estradiol and NADH[d,e]	0.013

[a] Concentration of inhibitor was 20 μM in all cases. [b] Values shown are for the mixture of diastereomers of **3**. All lines are least-square fits with $r \geq 0.992$. [c] k_{app} values (min^{-1}) for **3a** and **3b** were 0.026 and 0.021, respectively. [d] Concentrations of 17β-estradiol and 17α-estradiol were 32 μM (~5-fold K_m and ~5-fold K_i, respectively). [e] Concentration of cofactors was 150 μM. [f] k_{app} values (min^{-1}) for **3a** and **3b** were 0.014 and 0.011, respectively.

Scheme I[a]



5 ^{19}F NMR:[9] δ = 50.79

6 δ = 57.94, d, J = 8.3 Hz

7 δ = 65.02, s

[a] R = t-Bu.

To gain insight into the chemistry that could occur during affinity labeling by the trifluoromethylacetylenic alcohols, we investigated the nucleophilic addition of thioethoxide to the more readily prepared trifluoromethylacetylenic alcohol **5** under protic and aprotic conditions. The formation of regioisomeric products **6** and **7** in a 65:35 ratio was observed in both cases (Scheme I).[9,16] This indicates that covalent modification of estradiol dehydrogenase by steroidal trifluoromethylacetylenic alcohol **3** could involve reaction of an enzyme nucleophile with either of the acetylenic carbons in **3**. Since products that would be expected from an SN_2' displacement of fluoride were not observed, our model study suggests, but does not prove, that enzyme inactivation may not occur by this mechanism. Also, the propargylic alcohol group is not likely to be involved in the reaction since (trifluoromethyl)acetylene itself can undergo nucleophilic addition.[12] A more detailed examination of the reaction using additional trifluoromethylacetylenic alcohols and nucleophiles under a variety of conditions is therefore in progress.

In conclusion, we have shown that trifluoromethylacetylenic alcohols **3a** and **3b** are irreversible, active-site-directed inhibitors of estradiol dehydrogenase. We suggest that the (trifluoromethyl)acetylene functionality will be useful as a general affinity labeling group for enzymes and receptors. Since significant differences between the fluorine chemical shifts of alcohol **5** and the regioisomeric covalent adducts **6** and **7** are observed, the use of ^{19}F spectroscopy to study the structure of (trifluoromethyl)-acetylene-labeled enzyme adducts is also an attractive

(11) Time-dependent inactivations were carried out as described in ref 5 except that glycerol was excluded from all incubations since it caused decomposition of the inhibitor and 1 M carbonate buffer (pH 9.2) was used.
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(13) Turnover experiments indicated no detectible turnover of **3a** or **3b** in the presence of NAD^+ (200 μM). Protection by NAD^+ suggests that enzyme inactivation by either **3a** or **3b** cannot occur in evolutive ternary complexes. This result parallels our previous studies showing that acetylenic ketones do not inactivate hydroxysteroid dehydrogenases in evolutive complexes with NADH and possible reasons for the protective effects of pyridine nucleotides have been discussed.[5,14]
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(16) Regioisomers **6** and **7** were separated by HPLC using an Alltech (#60085) silica cartridge column (250 mm × 4.6 mm). Ethyl acetate (4%) in hexanes was used as eluent at a flow rate of 4 mL/min. Retention times for **6** and **7** were 6.5 and 7.5 min, respectively.

potential use for this affinity labeling group.

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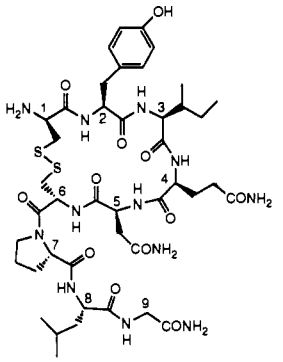
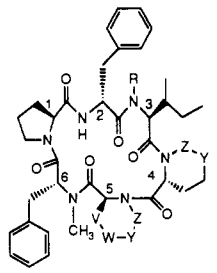
Receptor Ligands Which Bind the Oxytocin Receptor with Selectivity and High Affinity. Chemical Modification of a *Streptomyces silvensis* Derived Cyclic Hexapeptide

Oxytocin (OT) is a neurohypophyseal hormone which has been ascribed a pivotal role in parturition.[1] Evidence has accumulated to support the concept that the uterotonic action of OT and its stimulation of uterine prostaglandin release are events which combine to initiate labor.[2,3] Moreover, OT mediates the postpartum function of contracting the mammary myoepithelium to elicit milk letdown[4] and has also recently been implicated as a key element in preterm labor.[5,6]

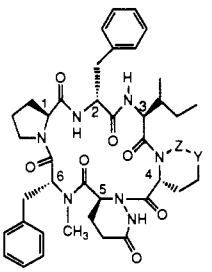
Since the breakthrough synthesis of OT three decades ago, considerable research has been devoted to the design of antagonists of this peptide hormone.[7,8] Interest in such compounds derives from the prospect of their use as novel therapeutic agents for the prevention of premature birth. While many promising in vivo antagonists of OT have been discovered over the years, the tactics for achieving this objective have generally been limited to modifications of the native OT and the closely related arginine vasopressin (AVP) structures.[3,5,7-14] One notable example is the development of the competitive OT antagonist 1-deamino-2-D-Tyr-(OEt)-4-Thr-8-Orn-oxytocin.[15,16] Initial clinical evidence suggests that this peptide is efficacious in the inhibition of uterine contractions in premature labor in humans.[6,17]



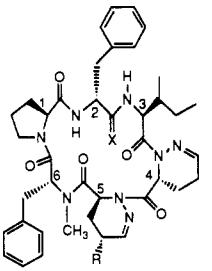
Oxytocin



(1) R = OH; V-W = CH_2-CH_2, Y-Z = CH_2-NH.
(2) R = H; V-W = CH_2-CH_2, Y-Z = CH_2-NH.
(3) R = H; V-W = CH_2-CH_2, Y-Z = CH=N.
(4) R = H; V-W = CH=CH, Y-Z = CH=N.
(6) R = H; V-W = CH_2-CH_2, Y-Z = CH_2-NCH_3.



(5) Y-Z = CH=N.
(7) Y-Z = CH_2-NH.



(8) X = O, R = $CH_2N(CH_3)_2$.
(9) X = S, R = H.
(10) X = H_2, R = H.

We recently reported the discovery of an entirely new structural class of OT antagonist, derived from a microbial source, and represented by **3**.[18] Despite the attractive OT/AVP antagonist properties of this compound (Table I), more potent and selective analogues with improved aqueous solubility[19] suitable for intravenous administration are required in order for compounds of this structural type to have practical utility. This communication represents our initial disclosure on chemoselective and regioselective transformations carried out on **3** with the aim of optimizing its physicochemical and pharmacological profile.

A key to the improved potency of **3** compared to its parent fermentation product **1** is oxidation of the piperazic acid (Piz) residues at positions 4 and 5. One hypothesis for this improved OT receptor affinity holds that changes in the conformation of these residues, brought about by the introduction of unsaturation, in turn influence the bioactive conformation of the 18-membered cycle. Ex-

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(19) The solubility of **3** in water at pH 6–8 is 68 μg/mL.

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