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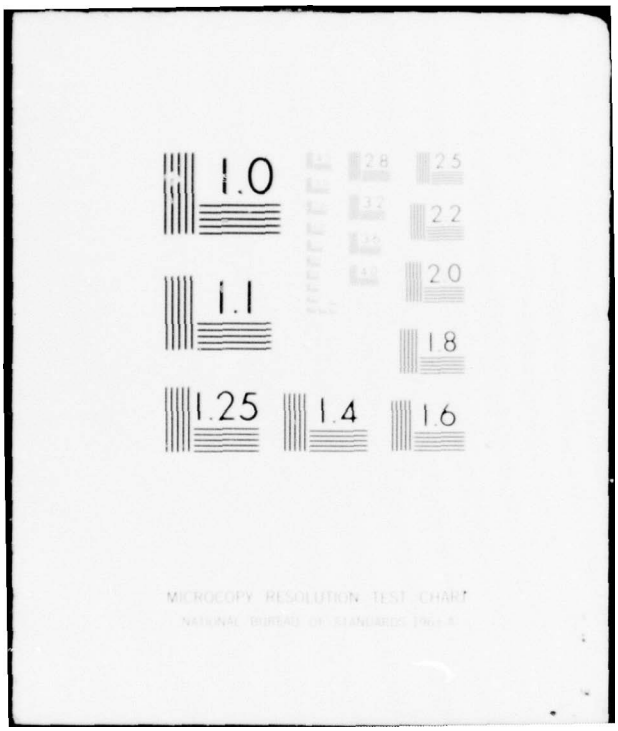
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Annual Report 2 and Final Report

Design, Synthesis and Pharmacology of Fluorescent Narcotic Analogs

Annual Report Covering October 1, 1974-
February 28, 1976

By

Chester M. Himel

W. Ben Iturrian

Arthur Yi

June 1976

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

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University of Georgia

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Fluorescent moieties can be synthesized into narcotic structures with retention of analgesic activity. Substituted acridines are a prolific source of fluorescent groups which can be synthesized by reasonable methods and introduced into narcotic structures. Favorable spectral properties include the quantum yield of fluorescence, the wavelength of excitation and emission and the extinction coefficient of absorption. Preliminary pharmacological data are reported.

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Summary

Fluorescent acridine moieties can be synthesized into narcotic structures related to meperidine and fentanyl. Substituted acridines are a prolific source of fluorescent narcotic-type structures with widely varying physical and spectroscopic properties. A wide range of synthesis methods are available by which favorable spectroscopic properties can be introduced into narcotic structures. Such spectral properties include the quantum yield of fluorescence, wavelength of excitation and emission and the extinction coefficient of absorption. Preliminary pharmacological data are reported for meperidine and fentanyl derivatives.

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Statement of Problem

The synthesis, pharmacological, biochemical and spectral study of fluorescent narcotic analogs and their substrate-system interactions is a new approach to the study of narcotic action and addiction mechanisms. Since most narcotic drugs are either non-fluorescent, or have inadequate spectral properties, optimum use of fluorescent methods requires the synthesis of narcotic analogs designed to incorporate favorable spectral properties into narcotic analogs. A key requirement of such synthetic, fluorescent analog molecules is retention of favorable biological activity within the narcotic moiety. The rationale for such an approach to the study of narcotic systems lies in the fact that fluorescent substrate analogs have been widely used in the study of enzyme, neural and other biological systems. Thus, there exists a major background in the synthesis and application of fluorescent analogs to biochemical problems (1). That background is available as a starting point for this preliminary study of fluorescence methods in research on narcotic mechanisms. Narcotic mechanisms are complex and their study involves major experimental problems. Thus, there is a need for new research methods such as fluorescence, to augment presently available research methodology.

Fluorescent substrate analogs are designed, fluorescent molecules which can act as alternative substrates for biosystems. Their synthesis and application is a biochemical and pharmacological problem involving stereochemical, chemical, structural, solubility, binding and spectroscopic factors. All such experimental parameters contribute to the biological responses and ultimate utility of substrate analogs.

The major problem studied in year one was a survey of design parameters for potential narcotic analogs, synthesis of candidate molecules and their intermediates and preliminary pharmacological testing. The critical question answered in year one was that fluorescent moieties could be incorporated into narcotic structures with retention of biological activity, in this case, retention of analgesic action.

The major thrust of research in year two was the design and synthesis of molecules having increased biological activity, a favorable hydrophilic-lipophilic balance, and increased aqueous solubility. These were determined to be limiting factors for the fluorescent analogs synthesized and studied in year one. Preliminary pharmacological studies were continued in year two. Spectroscopic binding studies were initiated on model substrate-system binding, since narcotic receptor preparations were not readily available.

Background

Fluorescent substrate analogs, fluorescent probes and other fluorescent molecules have a wide utility as visual, microscopic and spectral detection systems. In most cases, the application of fluorescence techniques requires the synthesis of designed fluorescent substrate molecules to meet the biochemical requirements of the system under study (2). Most biologically active substrates are non-fluorescent or have spectral properties inadequate to meet requisite experimental parameters. The ultimate success of fluorescence research is therefore dependent on the successful introduction of suitable fluorescent moieties into the drug structure without affecting activity and mode of action. In the case of enzyme substrates, successful synthesis of fluorescence into substrate molecules is related to the known fact that tolerance exists even though the active site may have specific and relatively circumscribed chemical and stereochemical requirements. Structure-activity data for most drugs indicate that "exo" area bulk tolerance exists in many drugs and that only a relatively limited part of the molecule has rigid stereochemical and chemical requirements.

The ultimate potentials from the use of fluorescent narcotic analogs in the study of narcotic mechanisms stems from their potential for detection in neural systems. They can be detected grossly by visual observation, or more precisely by fluorescence microscopy. Finally, they have the potential for detection of binding characteristics at receptor sites by measurement of spectral changes. If the ability to measure equilibrium binding exists, then a fluorescent narcotic analog can be used in a wide range of studies involving competition with known, active, non-fluorescent narcotic molecules. Thus, fluorescent analogs have the potential for utility per se, as well as utility as a measure of competitive effects in known binding systems.

)rescent drug analogs can be designed to be excited directly by irradiation or ectly by dipole-dipole energy transfer, a form of sensitized fluorescence. Dipole-dipole energy transfer occurs when an electronically isolated donor and acceptor are less than about 50 A distant, there is overlap in their emission and excitation spectra, and mutual orientation factors are met. When energy is transferred, the acceptor moiety is excited with concomitant quenching of the donor. Energy transferred is a sixth power function of the distance between the donor and the acceptor, therefore its transfer can be a spectral ruler. Dipole-dipole energy transfer can be significant in drug transport and receptor site studies, if the molecular structure of the transport molecule or the receptor site contains tryptophan moieties. Tryptophan is excited at 280 nm and emits in the range of 330 (depending on its environment). If the designed fluorescent drug analog has an excitation maximum at or near 330 nm, its excitation spectra overlap with the emission of tryptophan can allow dipole-dipole energy transfer, all other conditions being met. In some cases, this would be a favorable spectroscopic result, in other cases, it may be valuable to design the fluorescent moiety to have an excitation maximum well removed from the emission of tryptophan moieties. Thus, fluorescent drug analogs can be designed to involve dipole-dipole energy transfer or to have no excitation

interaction from tryptophan in the biosite. Ultimate design parameters will have to be determined by research results.

Non-specific binding of substrates to biomolecules or systems can be a problem. Organic molecules, including various drugs often bind to hydrophobic areas on protein structures. Thus non-specific binding of organic solvents and their effects on proteins has been studied (3). Solvent effects on cholinesterase has been studied (4).

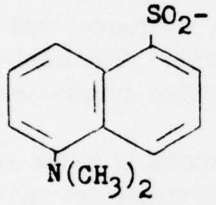
The general effects of structure on fluorescence spectra are usually what would be expected from the nature of chromophore-auxochrome interactions as determined by absorption data. Not all structural parameters which might affect useful fluorescence responses are known. The physical spectroscopy of fluorescent substrates has lagged behind their potential uses in biochemical research. Notwithstanding, a very considerable body of spectroscopic data exists. Reasonable synthesis decisions can be made.

The elegance of fluorescence techniques as research tools in the study of biochemical problems (particularly in mode of action, drug transport and receptor site research) lies in their wide scope and sensitivity. That sensitivity can be orders of magnitude greater than that available from absorption spectroscope. A major advance in the use of fluorescent molecules in <u>in vivo</u> research has stemmed from the availability of microspectrofluorometers. They are a fluorescence microscope which allows localization of the viewing site to very small areas. In addition, the excitation and emission wavelength are controlled and/or scanned by monochrometer systems. The fluorescent moiety can be studied by (1) its relatively precise location in the histological sample (2) its excitation spectrum (3) its quantum yield (4) its emission spectrum. Where spectroscopic factors are favorable, it is reasonable to expect that fluorescent moieties can be detected and studied in detail in the range of 10^{-9} to 10^{-11} grams. Limitations as to detection are related to (1) quantum yield of the fluorescent moiety (2) extinction coefficient of absorption (3) background interferences of other fluorescent molecules. To a major extent these problems can be met by known design data. In many cases, fluorescent molecules act as fluorescent probes, that is, their spectral responses are sensitive to their environment. This effect can be a valuable design parameter.

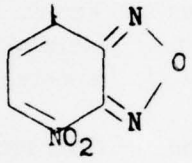
Useful fluorescence is nearly always associated with the pi electron systems of unsaturated or aromatic molecules. It can be derived from aromatic rings or from linear or cyclic conjugated double bond systems. The 5-dimethylaminophthalene-1-sulfonyl (Dns-) moiety (I) has a fluorescence which reflects its aromatic naphthalene ring system as modified by the dimethylamino and sulfonyl auxochrome groups. The 1-5 relationship of these groups is spectroscopically significant (5). The dansyl moiety is readily introduced into substrate molecules which contain aromatic or aliphatic amines (to give the corresponding sulfonamide). We have found that most Dns-sulfonates are photolytically unstable, although a few phenolic sulfonates appear

to be stable (2). Most Dns derivatives have an excitation maximum near 330 nm and emission in the 500-580 nm range.

The NBD moiety (II) was introduced by Ghosh and co-workers (6) and appears to be a uniquely valuable fluorescent moiety for introduction into designed substrate analogs. The 7-chloro in NBD chloride is reactive with amines, alcohols and mercaptans to give stable, fluorescent derivatives at the 7 position. The excitation maximum is in the range of 464 nm with emission in the range of 500-525 nm.



I



II

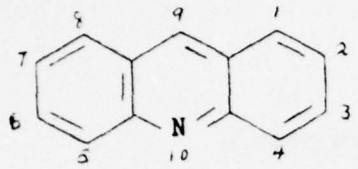
Where molecular size is critical, useful fluorescence in the 360 nm range is available from introduction of p-aminobenzoyl groups. Naphthalene and indole rings have relatively comparable fluorescence properties with excitation near 280 and emission near 330 nm. The objectives of this research are not involved in modification of the stereochemical center of the drug molecule. Emphasis is therefore placed on introduction of suitable fluorescence moieties into the molecule at sites where bulk does not appear to be a major factor.

The effect of multiple binding sites in substrate-biosite binding processes is discussed by Himel and Chan (1). It would be expected that the fluorescent moiety may either have no effect on the binding of a fluorescent narcotic analog or that the fluorescent moiety may become stereochemically available for hydrophobic area binding. It is doubtful if the effect of structure on binding can be predicted in advance.

For optimum utility, fluorescent drug analogs should have a fluorescence emission in the range of 300-600 nm and a quantum yield above about 0.5 when complexed during transport or at the receptor site. If the fluorescent moiety acts as a fluorescent probe when bound, its spectral changes will reflect the environment of the site to which it is bound. In some cases, those spectral changes may include total quenching of the fluorescence. A result which may or may not be desired and may require molecular modification.

The acridine ring system is a prolific source of fluorescent, biologically active drugs (1). Derivatives at the 2, 3, and 9 positions are readily prepared and offer

a wide range of physical, chemical, biological and spectroscopic variations.



Acridine

Ex 325 (CH_3OH) corrected

Em 425 " "

III

Because of the many favorable properties associated with the acridine system, we have placed initial emphasis on the synthesis and testing of fluorescent narcotic analogs containing the acridine ring system. Preliminary pharmacological data obtained included evaluation of analgesic activity and other pharmacological effects. The spectroscopic part of the research was concerned with determination of the interrelationships between substrate and spectral effects, as they relate to biological activity.

Approach

The initial research was concerned with the design and synthesis of fluorescent derivatives of normeperidine (norpethidine). The amino moiety of the piperidine ring was linked by a C_2 or C_3 alkylene bridge to a fluorescent group. The fluorescent moiety included acridine and dansyl derivatives. Our prior biochemical and spectroscopic research has shown the potential importance of the acridine ring system. It has, (a) favorable fluorescence emission in the range of 450-550 nanometers (nm), (b) quantum yields in excess of 0.5, (c) favorable extinction coefficients of absorption, (d) useful solubility characteristics. Generalized structures are given in Figure I.

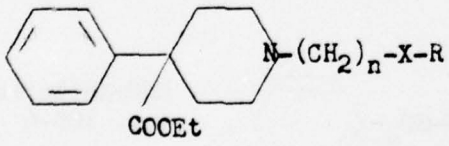


Figure I

where R = 3, or 3-6-substituted acridine, 1-naphthyl or phenyl

n = 2 or 3

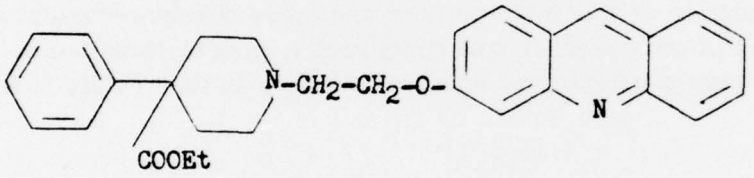
X = O or $-\overset{\overset{O}{\|}}{C}-N-$ H

Synthetic methods were largely based on available reviews of acridine chemistry (7) (8). The application of new synthesis methods was also studied, particularly ring construction by the Friedlander and Pfittzinger synthesis.

Results

I. Fluorescent Narcotic Analogs Synthesized (year 1)

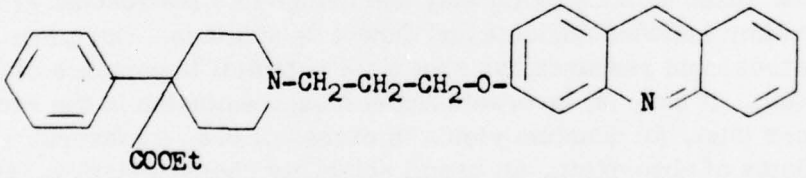
In the first year, three fluorescent derivatives of normeperidine (norpethidine) were prepared and subjected to preliminary pharmacological testing. Two of the new compounds contained a C_2 and a C_3 methylene bridge to 3-oxyacridine while the third fluorescent analog was a dansyl derivative of anileridine. Structures are as follows:



AD II

m.p. 118-19°C.
Ex (max) 350 nm (corr)
Em (max) 470 nm (corr)
M.W. 445

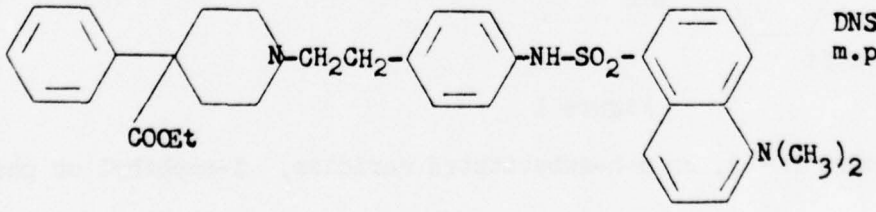
IV



ADIII

m.p. 130-1
Ex (max) 350 nm (corr)
Em (max) 470 nm (corr)
M.W. 459

V



DNS-Anileridine
m.p. 105-6

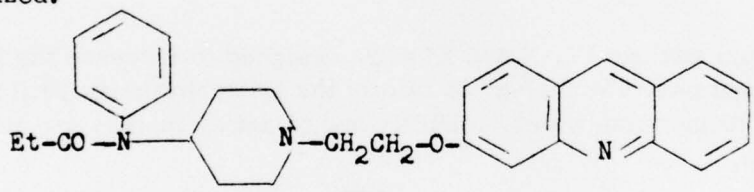
VI

2. Fluorescent Narcotic Analogs Synthesized (year 2)

In year two, emphasis was placed on synthesis and study of fluorescent narcotic analogs having increased activity, increased water solubility and more favorable hydrophilic-lipophilic balance. Major difficulties were encountered in year one in the development of formulations of the drugs which would meet dose, stability, and other biological in vivo considerations. Increased activity decreases any requisite dose. Increased water solubility limits formulation problems. ADII and ADIII were noted to be highly lipophilic and that they accumulated rapidly in fatty tissues. This was a complicating factor relative to measurement of "effective dose" and in vivo activity.

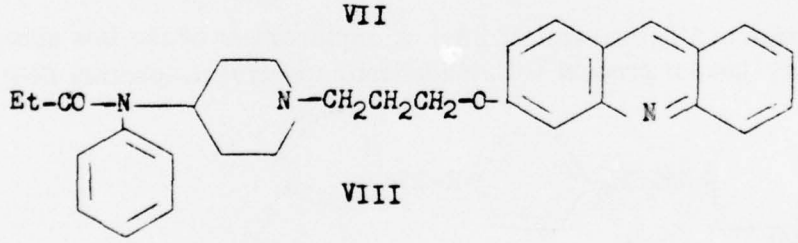
Increase in activity was attempted through the replacement of nor-meperidine with nor-fentanyl in the synthesis of analogs. Modification of the hydrophilic-lipophilic balance was studied by an increase in the hydrophilic nature of the acridine moiety. This was done by replacing the 3-ether function with a 3-amido, 3-6 amido or 3-amido-6 amino moiety. Synthetic routes to 3-hydroxy-9-carboxy acridine were also studied, and an effective synthesis of this intermediate was evolved.

Since year one, the following fluorescent narcotic analogs have been synthesized.



AFII

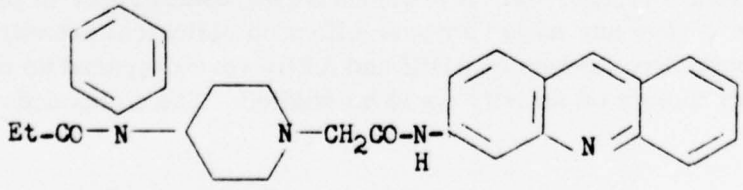
VII



AFIII

VIII

Fluorescent narcotic analogs VII and VIII replace the meperidine moiety of IV and V, with a fentanyl group.



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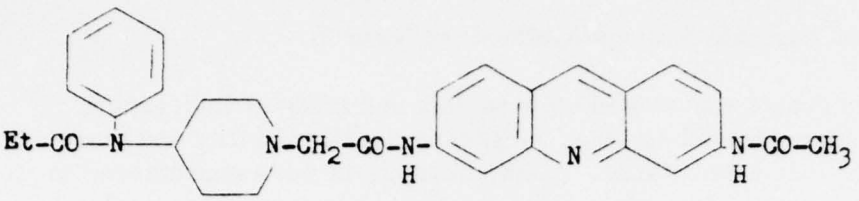
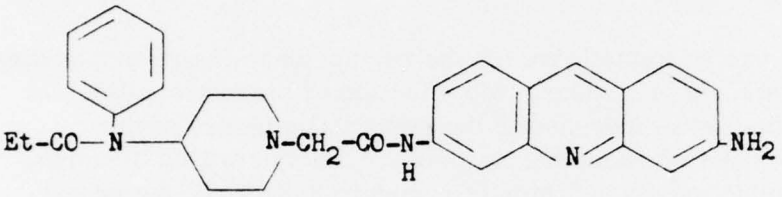


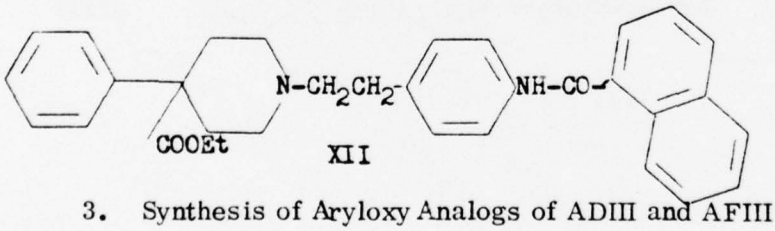
X



XI

Fluorescent fentanyl analogs IX, X and XI were designed to increase the hydrophilic nature of fluorescent narcotic analog, to modify the hydrophilic-lipophilic balance of the molecules, to increase water solubility and to act as models for molecular design and activity.

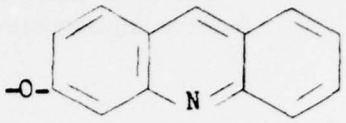
Modification of VI (Dns-anileridine) by replacement of the Dns group with a naphthoyl moiety gave a product with inadequate spectral properties (XII).



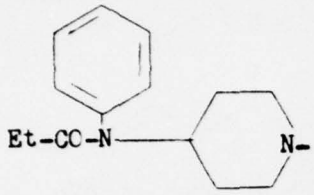
XII

3. Synthesis of Aryloxy Analogs of ADIII and AFIII

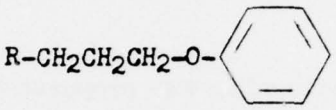
The 3-oxyacridine moiety (XIII) has proven to be a very interesting group for introduction into narcotic structures. It provides highly useful spectral properties and does not appear to have any major adverse effect on biological activity. Phenoxy and 1-naphythyloxy analogs of ADIII and AFIII were prepared so that relative effects of the aryloxy moiety on activity could be studied. The compounds synthesized were:



XIII

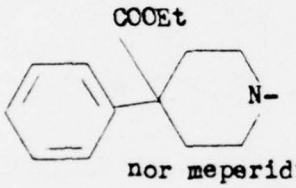


nor fentanyl

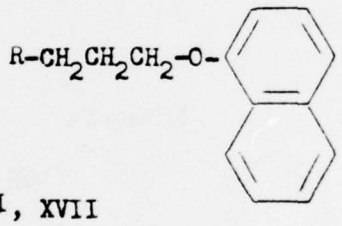


R = nor fentanyl or nor meperidinyl

XIV, XV



nor meperidinyl



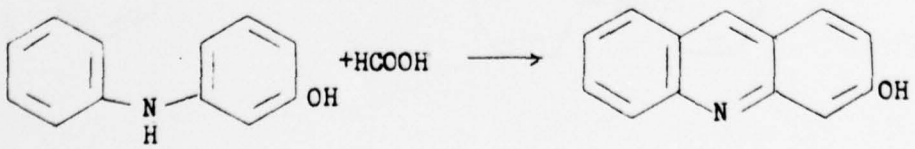
R - nor fentanyl or nor meperidinyl

XVI, XVII

The relative biological activity of these model compounds has not been obtained at this time.

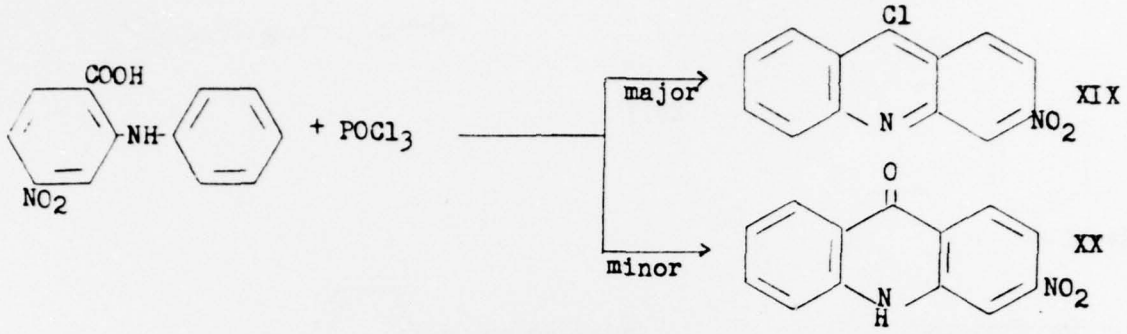
4. Fluorescent, Intermediate Compounds

1. 3-Hydroxyacridine was prepared by ring closure of m-hydroxydiphenylamine with formic acid in glycerol at 180°.

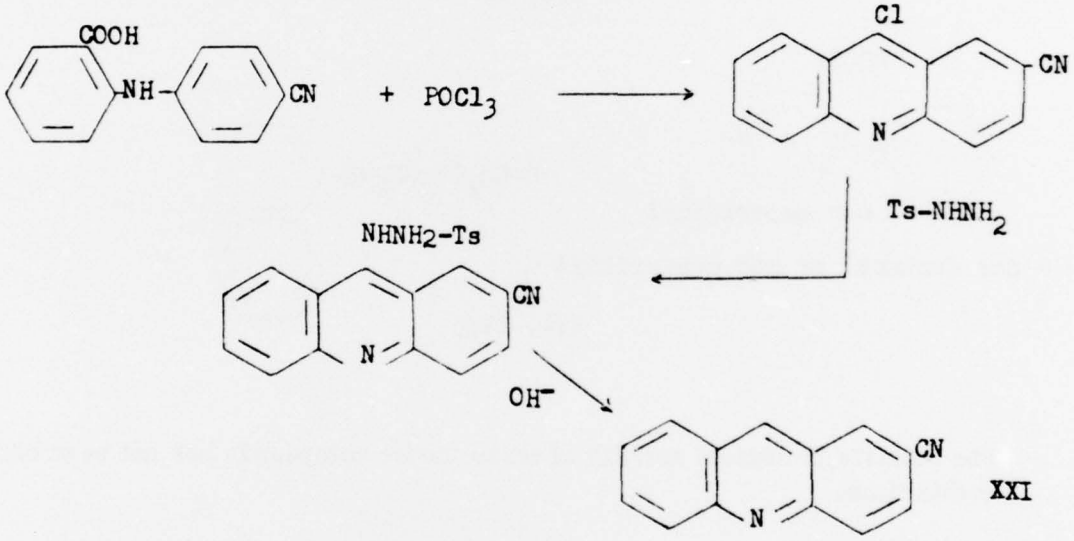


XVIII

2. 9-Chloro-3-nitro acridine was prepared by ring closure of 2-carboxy-5-nitro diphenylamine with phosphorous oxychloride.

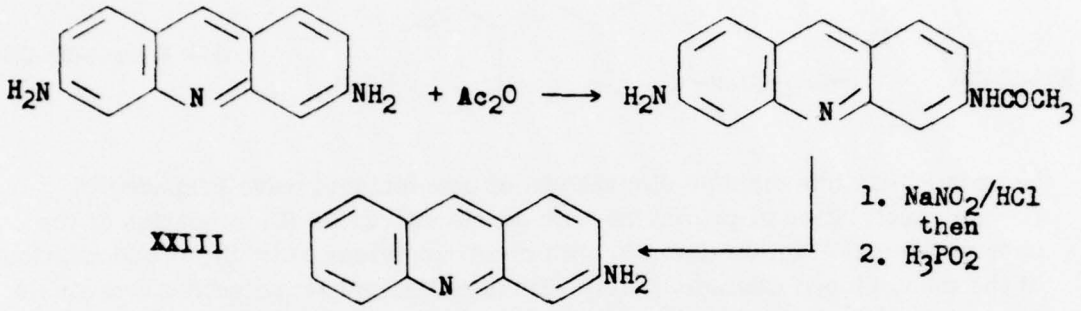


3. 2-Cyanoacridine was prepared by ring closure of 2-carboxy-4'-cyano diphenylamine with phosphorous oxychloride and the 9-chloro atom can be removed by alkylation with p-tosyl hydrazine followed by base hydrolysis.



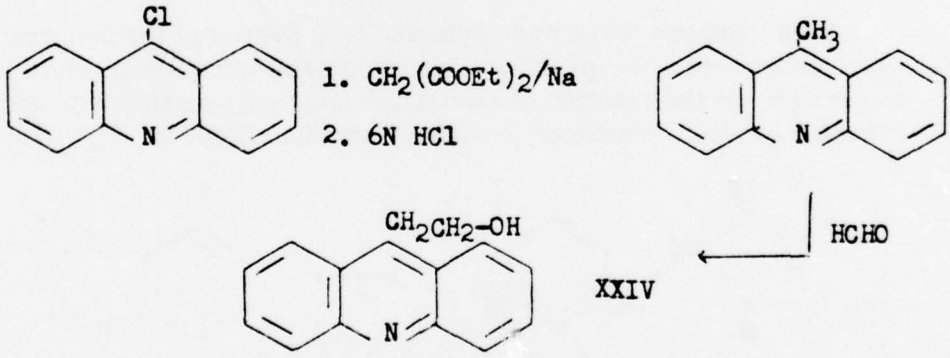
4. 1-Methyl acridone was prepared by ring closure of 2-carboxy-3'-methyl diphenylamine and phosphorous oxychloride.

5. The synthesis of 3-aminoacridine from ring closure of 2-carboxy-5-nitro diphenylamine followed by reduction of nitro group was unsuccessful. Catalytic hydrogenation (Pd/C) lead to dimerization or polymerization. The synthesis of 3-aminoacridine in this research was based on 3,6-diaminoacridine. After monoacetylation with acetic anhydride to gave the monoamide, diazotiation of the free amino group followed by hypophospherous hydrolysis gave 3-aminoacridine.



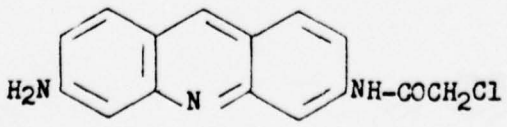
XXIII

6. B-(9-Acridinyl) ethanol was prepared by reaction of ethyl malonate anion with 9-chloroacridine followed by decarboxylation to give 9-methyl acridine. The methyl group on 9-position is very acidic. It reacts with formaldehyde to give B-(9-acridinyl) ethanol.

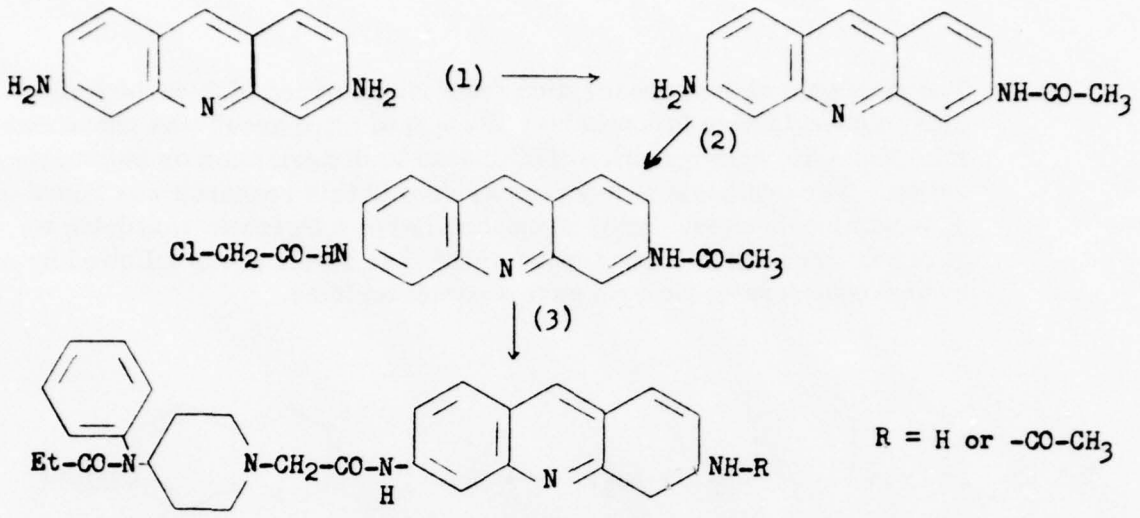


XXIV

7. The synthesis of 6-amino-3-(2-chloro-1-oxo) ethylamino acridine from proflavine reacted with chloroacetylchloride was unsuccessful. The proflavine itself serves as base. Di-alkylation occurs instead of monoalkylation. Using DCMA as scavenger, the major product was monoamide. This monoamide was unstable and easily polymerized.



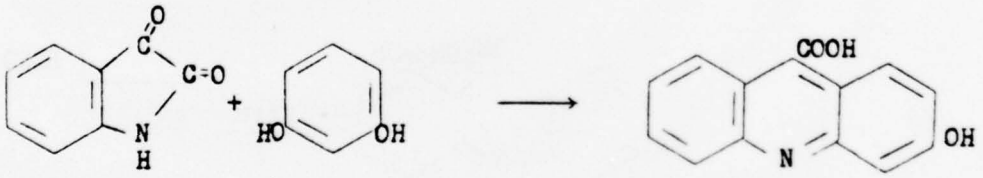
XXV



6-Amino-3-amido acridine derivatives of nor-fentanyl were prepared by (1) monoacetylation of proflavine with acetic anhydride (2) formation of the unsymmetrical diamide (from 1) with chloroacetylchloride (3),—Condensation of the mono chloro diamide moiety with nor-fentanyl (4) selective hydrolysis (removal) of the 6-acetamide group (if the free 6-amino group is desired instead of the 6-acetamido moiety).

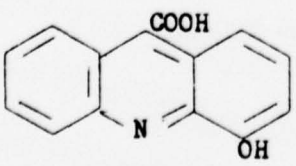
5. New Synthesis Methods for Acridines

This synthetic work was prompted by a survey of the preparative literature which uncovered a few previously unrecognized innovations. In a little known publication (9) the reaction of resorcinol and isatin under basic conditions was reported to yield 3-hydroxy-9-carboxy acridine XXVI in 76% yield. Repetition

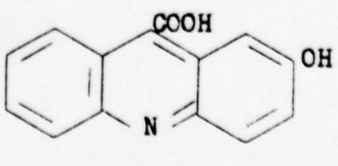


XXVI

of this work brought similar results in our hands. The solubility characteristics of (XXVI) are advantageously different than those of 3-hydroxyacridine which has been the fluorescent tag workhorse in this study. Studies pursuant to the incorporation of (XXVI) into the drug-fluorescent tag are in progress. In similar fashion catechol and hydroquinone can be condensed with insatin yielding XXVII and XXVIII respectively.

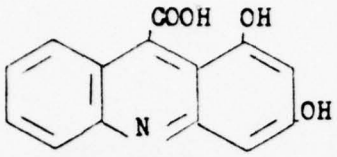


XXVII



XXVIII

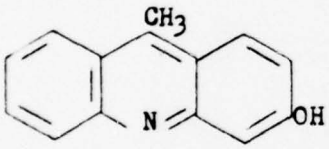
The condensation (9) (10) of sym 1,3,5-trihydroxybenzene and isatin is also known although we have had little interest in the product at this time.

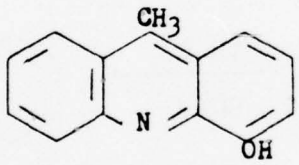


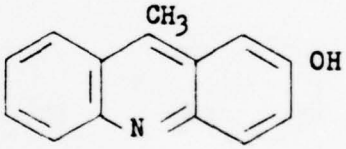
XXIX

The earlier work (9) claimed that catechol and hydroquinone did not undergo this condensation when the base employed was potassium hydroxide. From the present knowledge of ambident nucleophiles (11) it can be generally stated that C-alkylation at the ortho position to a p enol can be made to occur in concentrated polar solutions where the cation of the base employed is quite small. By altering the base composition from potassium to lithium hydroxide the hitherto unreported condensations were shown to be possible.

Similar condensation products are available from the reaction of o-aminoacetophenone and the corresponding phenol i.e., resorcinol, catechol, hydroquinone respectively.

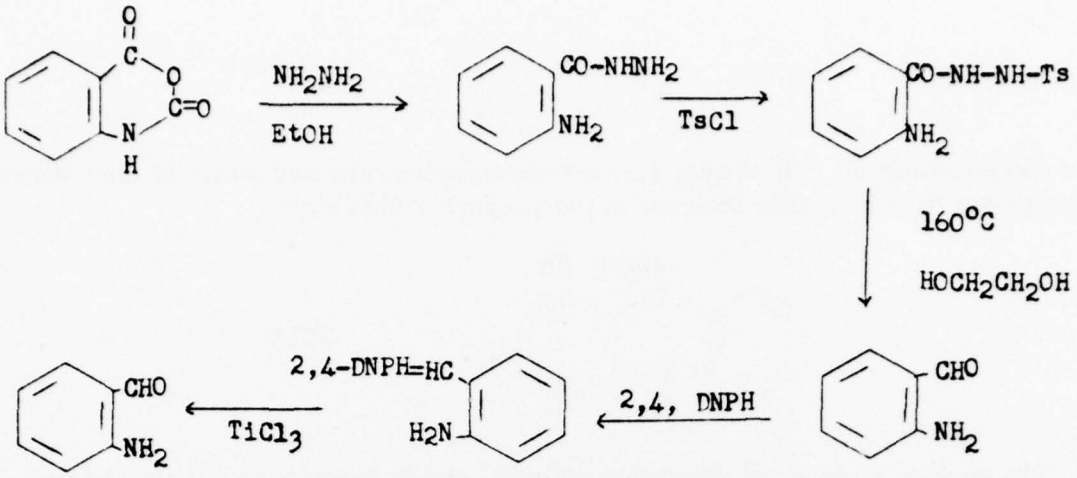






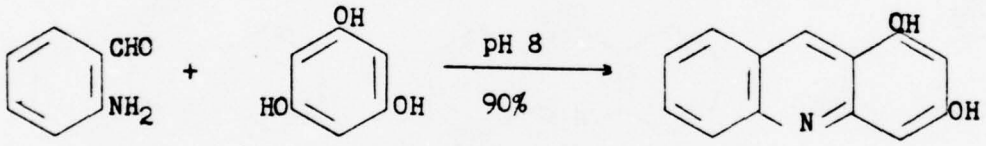
This reaction provides alkyl or aryl substitution at position 9 on the acridine nucleus depending on the aromatic amino ketone employed.

Condensation reactions leading to a hydrogen substituent at position 9 occur in a similar manner by employing o-aminobenzaldehyde. The preparation involves reduction of o-nitrobenzaldehyde followed by steam distillation. Very little can be said in favor of this experimental procedure. An alternative method to synthesize o-aminobenzaldehyde from anthranilic acid in 35% yield via a McFadyen-Stevens rearrangement on the corresponding aroyl p-toluenesulfonyl hydrazide is available. We have found that isatoic anhydride is a much more convenient starting material for this synthesis. But more important is the finding that the yield of reaction can be significantly raised (to ca 50-60%) by isolating the o-aminobenzaldehyde as the 2,4-dimtrophenylhydrazone and then cleaving the hydrazone with titanium (III) chloride (12).



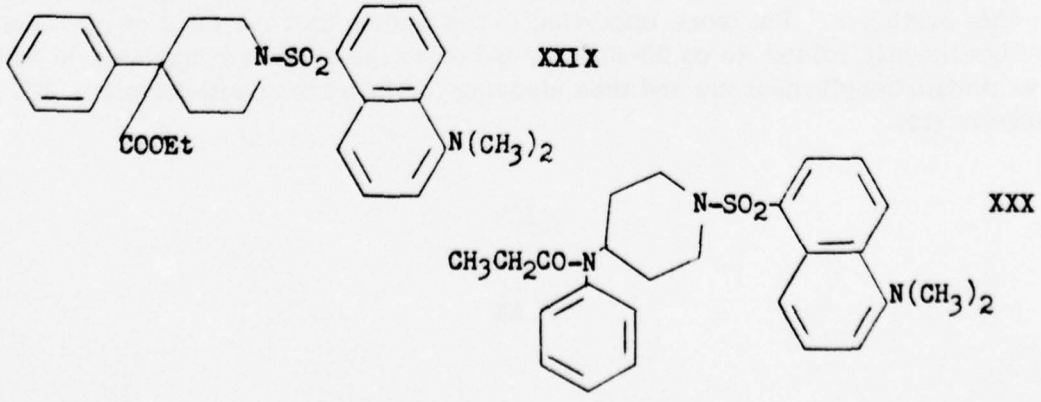
This method provides a purified product and a convenient storable source of the aminobenzaldehyde.

The condensation of o-aminobenzaldehyde with the phenols previously employed has yet to be effected but the condensation with sym-1,3,5 trihydroxybenzene has been reported (13).



6. Compounds for Analytical Purposes

Fluorescent dansyl derivatives of nor-meperidine (XXIX) and nor-fentanyl (XXX) were prepared for use in the study of potential metabolic products of the fluorescent narcotic analogs used in this research. In particular, these compounds were prepared as model compounds to determine whether nor-meperidine and nor-fentanyl were formed as metabolic products of these analogs in vivo and to study the presence of nor-meperidine as a metabolic product of meperidine by N-demethylation processes.



7. Fluorescence Data

3-Hydroxyacridine has an excitation maximum at 450 nm and an emission maximum at 550 nm (corrected). Conversion of the hydroxyl to an ether gives a blue shift with excitation in the range of 350 nm and emission in the range of 450 nm. As ethers of 3-hydroxyacridine, ADII (compound IV), ADIII (compound V), AFII (compound VII) and AFIII (compound VIII), have substantially identical fluorescence spectra. The presence of the methylene bridge isolates the acridine moiety from any contribution by the fentanyl or meperidinyl group.

If metabolic cleavage of the ether linkage occurs to produce 3-hydroxyacridine, it would be readily detected in the presence of the parent molecule. If N-dealkylation occured to produce normeperidine or norfentanyl, we have shown that reaction of the brain homogenate with dansylchloride produces the dansylmeperidine or fentanyl which can be separated and detected in the presence of amino acids and their dansyl products.

All of the acridine compounds prepared in this study share a common characteristic of intense fluorescence. Emphasis was placed on acridine derivatives in this study because of their excellent spectroscopic properties and relative ease of synthesis methods. Change in physical properties of the narcotic analog are possible by change in structure of the acridine moiety used. This allows considerable flexibility in ultimate design of useful molecules.

The fluorescence of ADII, ADIII, AFII and AFIII makes it possible to use these analogs in <u>in vitro</u> and <u>in vivo</u> detection systems. Their distribution in the body has been observed to be highly selective and easily noted. Small interperitoneal doseages cause intense fluorescence in the fat, brain and blood vessels of the animal. A blue fluorescence was observed in fatty areas while a green fluorescence was observed in the cortical area.

8. Pharmacological studies (year 1)

The objective of the preliminary study in year one was to determine whether fluorescence could be synthesized into a narcotic analog with retention of analgesic activity. This was accomplished. Selected analogs did show analgesic potential. However, the analgesic potential demonstrated was at a low level of potency. Synthesis of structures with increased potency is a reasonable potential since it is well known that normeperidine and norfentanyl derivatives reported in the literature have major variability in antinoceptive potency, ranging from 0.1 to over 200 times that of morphine.

Major compounds produced were subjected to a variety of tests to determine physical solubility, stability and pharmacological-toxicological properties. A summary of the pharmacological-toxicological properties of fluorescent analogs from year one are indicated in Table I. The following test methods were used:

a. Analgesia Testing

Analgesic activity was estimated by the antinociceptive method of Eddy and Liemback (J. Pharmacology Exptl. Therap. 107: 385, 1953). Groups of 6 CF1 mice were randomly assigned to treatment and the reaction time determined 15 and 1 minute before injection. The agents were injected subcutaneously (.01 or .02 cc/gm of injections vehicle) and the reaction times redetermined at 20, 40, 60, 90, 120 and 180 minutes by 2 "blind technicians." The reaction time for the mice to show a nociceptive response characterized by vigorous licking of their hind paw was recorded and the mean value for experiments and technicians determined. Mice which did not respond within the arbitrary chosen time of 30 sec were removed from the hot plate and this value was taken as 100% inhibition of the nociceptive response. The mean reactive time for the vehicle control groups was averaged ($10.5 \pm .5$) was taken to represent 0% inhibition. The average increase in reaction time was calculated for each post-injection period. Statistical significance was tested using the student's t-test. Treatment groups possess a significant ($P=0.05$) increase in reaction time the RT_{50} (AD_{50}) values and their 95% confidence limits were estimated by Probit analysis (Table 2).

The solvent required for the analogs (30% propylene glycol, 2% benzyl alcohol, 1% ascorbic acid in aquous solution, .02 cc/gm body weight) caused a slight alteration in the reaction time to $10.5 \pm .5$ sec. A slight alteration in the dose response and duration profile for meperidine was also noted. ADIII was observed to possess approximately 0.1 the analgesic potency of meperidine. Peak analgesic activity appears more slowly than with meperidine but onset of action is a function of dose. ADII and the dansyl derivative of anileridine appear to be inactive as analgesics. The dansyl anileridine appears to be more toxic than the acridine analogs.

The acridine compounds are extremely fluorescent. The body fat, brain and blood vessels are highly fluorescent following small subcutaneous injections of ADIII. The time course of the fluorescence in the brain presents a most promising drug tagging device since administration of a threshold analgesic dose (.17 mm/Kg) results in a marked concentration in the brain which disappears as the reaction times return to normal.

b. In vitro evaluation

The analogs share meperidine antispasmotic activity in rabbit ileum stips treated with physostigmine (Koppanyi and Karczmar Experimental Pharmacodynamic, p. 152, 1956) ADII was particularly potent. The compound appears to increase pendular movements in the strip--an effect that was demonstrated to be shared with the 3-OH-acridine. ADII also potentiates the effect of acetylcholine on the frog rectus, indicating cholinergic enzyme or receptor binding.

Shaw and Bentley (Aust. J. Exptl. Biol. 31, 573, 1953) made similar observations on other acridine compounds and concluded that acridine actions on cholinergic

systems does not reside entirely on anticholinergic effects.

c. Other effects

Meperidine is an extremely potent convulsant when injected ip or iv. A timed intravenous infusion threshold test for seizures was developed for meperidine using a modification of the Bastian et al method (J. Pharmacol. Expt. Therap. 127:75, 1959). Unfortunately the solvent required for the analogs markedly alters the threshold. 0.05 mM injected intravenous produces a maximal seizure independently of mouse body weight.

ADIII appears to be a convulsant with actions very similar to meperidine itself. No convulsions were produced by the 3-OH-acridine, Mayer and Bain (J. Pharmacol. Exptl. Therap. 112:210, 1954) reported that a 9-acridone was a powerful convulsant. 0.1 mM/Kg of ADIII caused a richly fluorescent cortex. The visible differences in uptake between various parts of the brain suggest possible histochemical use for the probe.

Table 1. Pharmacological Properties of Fluorescent Narcotic Analogs
Properties of Fluorescent Narcotic Analogs. (year 1)

Approximate LD_{50}	Meperidine	ADIII (V)	ADII (IV)	DNSA (VI)
ip in 10 gm mice	.06 mM/Kg	.22	.3	.1 .2
sc in 10 gm mice	.3	.7	.3	.2
Antinociceptive Activity (AD_{50} with 95% confidence limits, mM/Kg)				
(50% Inhibition of Response)	0.015 (0.14-.017)	0.16 (0.15-.18)		X[a]
(50% Increase in Reaction Time)	.012 (.011-.013)	.11 (.07-.19)		X
Onset of activity (min)	20	20	120	X
Peak activity (min)	20	40	X	X
Antispasmotic Activity (rabbit ileum)				
Effective Conc. to block 1:100,000 physostigmin	1:50,000	1:50,000	1:100,000	X
Seizure Threshold (timed iv infusion threshold, .03 mM/ml, .006 ml/sec)				
clonic (n = 4)	.17 ml	.22	X	X
tonic (n = 4)	.22 ml	.27	X	X

a) data not obtained.

Table 2. The antinocicpetive activities of fluorescent narcotic analogs. (year 1)

Agent[b]	Dose (mM/Kg s.c.)	No. Animals	Time after injection (minutes) 20	40	60	90	120	180
			Inhibition of Response (%)[a]					
Meperidine	.01	12	8	0	0	x[c]	x	0
	.03	6	100	67	67	x	67	17
	.06	12	100	92	92	x	75	33
	.11	18	100	100	83	50	x	39
AD III	.11	12	0	0	0	0	0	x
(V)	.17	12	17	33	58	25	17	8
	.22	12	25	75	50	58	42	25
AD II	.11	12	0	0	0	x	0	0
(IV)	.22	6	0	x	0	0	0	0
	.33	12	0	0	0	8	8	0
DNS-A	.11	12	0	0	0	0	0	0
(VI)			Average Increase in Reaction Time (sec)					
Meperidine	.01	6	5	-	-	x	x	-
	.03	6	20	16	16	x	14	7
	.06	12	20	20	18	x	14	12
	.11	18	20	20	19	15	x	8
AD III	.11	12	[d]		10			
(V)	.17	12	11	17	13	9	8	6
	.22	12	10	18	15	14	14	8
AD II (IV)	.33	12				6	6	
DNS-A (VI)	.11	12						

[a] Percent of mice not responding during 30 sec on a 59±.8 degree hot plate.

[b] agents prepared in a 30% propyleneglycol, 2% benzyl alcohol, 1% ascorbic acid-aqueous injection vehicle and injected subcutaneously with .01 or .02 cc/gm body weight.

[c] data not collected.

[d] no statistical increase from injection behicle reaction time of 10.5±.5 sec.

[e] not injected with control vehicle.

9. Pharmacological studies (year 2)

a. Evaluation of analgesic activity

Weanling mice (10 ± 3 gm) or adult female (23 ± 4 gm) of the CAW:CF1 mice were used. All the drugs were dissolved in 2% benzyl alcohol, 30% polyethyleneglycol 400 and g.s. with distilled water and injected at 0.01 ml/gm body weight subcutaneously over the scapula.

Analgesic activity was estimated by the antinociceptive method of Eddy and Liemback (1953) as modified by Johannesson and Woods (1964). The copper drum 14 inches in diameter and 81/4 inches in height was used to boil reagent grade acetone. The boiling point averaged 56.0 ± 0.1 °C and was constant from day to day. The reaction time as defined by Eddy was measured to nearest 0.1 seconds with an electric timer. Groups of 6 CF1 mice were randomly assigned to treatment and the reaction time determined twice at twenty minute intervals before injection. The agents were injected subcutaneously and then tested at 15 minute intervals for 75 minutes or until the reaction time returned to control levels. A summary of the analgesic activities and pharmacological-toxicological properties of the fluorescent analogs appears in Tables 3 and 4. AFIII (VIII), ADIII (V) and α-naphthoxy-FIII (VXI) possessed significant analgesic effect. The other analogs possessed no analgesic activity at subconvulsive and nontoxic dosage.

In these alkylether bridges, the 4 member bridge $-CH_2CH_2CH_2O-$, was a prerequisite for activity (V & VIII). The corresponding 3 membered bridge gave inactive substrates (IV & VII). The fentanyl analog (AFIII-VIII) appears to be 8-10 times as active as norfentanyl and twice as active as the meperidine derivative ADIII (V). Norfentanyl and normeperidine are possible metabolites of AFIII (VIII) and ADIII (V) respectively. Both normeperidine and norfentanyl possess convulsive activity but normeperidine also possesses significant analgesic activity. It is reasonable that the analgesic activity of AFIII is not the result of cleavage to norfentanyl, however, it is possible that the cleavage of ADIII to normeperidine could contribute to the analgesic effect.

The greater analgesic activity of AFIII in weanling mice was attributed to an approximate five-fold difference in plasma binding. A similar age dependent plasma binding for meperidine was recently reported (Clin. Pharmacol. Ther. 17:21-30, 1975). However, meperidine metabolism and excretion is also influenced by aging (Chan et. al., J. Pharm. Pharmacol. 27: 235, 1975).

The unexpected sharp decrease in analgesic activity as the dose of AFIII was increased from 22 um/kg to 107 um/kg in conjunction with the narcotic antagonist binding studies (vida infra) suggested that AFIII possesses mixed narcotic agonist and antagonist properties. A pilot study to assess this possibility met a limited degree of success (Table 5). The mixed agonist activity of AFIII is most evident 30 minutes

Table 3. The analgesic activity of narcotic analogs.

Agent	Dose (um/kg sc)	No. Mice	Average Weight (g)	Time after injection (minutes) 15	30	45	60	75
				Inhibition of Responses (%)[a]				
Norfentanyl	107	6	10 ± 3	0	0	0	0	x
	214	6	10 ± 3	13	13	0	13	x
	321	8	10 ± 3	13	13	13	0	x
	43	6	20 ± 3	0	17	17	0	0
	214	6	25 ± 3	33	13	13	13	x
	321[d]	6	26 ± 2	0[d]	0[d]	0[d]	x	x
DNSfentanyl (XXX)	107	6	10 ± 3	0	0	0	0	x
AFII (VII)	43	6	20 ± 3	0	17	17	0	x
	107	6	24 ± 1	0	0	0	0	x
AFIII (VIII)	22	12	18 ± 4	17	33	33	25	0
	43	12	18 ± 3	17	33	33	17	17[b]
	107	12	23 ± 4	8	17	17	0	x
	107	6	10 ± 3	17	50	50	33	x
α-napthoxy-FIII (XVI)	107	6	10 ± 3	0	17	33	33	x
phenoxy-FIII (XIV)	107	6	10 ± 3	0[b]	20	0	0	x
α-napthoxy-FIII. HCl	107	6	13 ± 2	0	0	0	0	x
phenoxy-FIII. HCl	107	6	13 ± 2	T[c]	17	0	17	x
ADIII (V)	220	6	10 ± 3	0	50	67	33	33[h]
DNS-anileridine (VI)	330(ip)	6	10 ± 3	0	0	T[f]	T[f]	T[f]
N-α-napthoic anileridine (XII)	330(ip)	6	10 ± 3	0[bc]	40	T[e]	T[e]	T
anileridine	5.9(ip)	6	10 ± 3	83	83	33	50	x
	11.80(ip)	6	10 ± 3	100	100	100	100	x
Meperidine	10	30	10 ± 3	30	13	0	0	x
	20	12	10 ± 3	33	33	0	0	x
	60	12	10 ± 3	100	42	42	42	3
	120	6	25 ± 3	100	100	83	83	50
normeperidine	220	6	12 ± 2	50	50[e]	50	T[e]	T[e]

[a] Percent of mice not responding during 30 seconds or a 56 ± .1°C hot plate
data not obtained.
[b] Lethal to one or more
[c] 50% lethargic and ataxic.
[d] 100% hyperexciteability, piloerection and marked increase in spontaneous activity.
[e] Clonic seizure and ataxia observed in one or more.
[f] 60% lethargic and ataxic 45 minutes postinjection, 100% at 60 and 75 minutes.
[h] 0% at 90 minutes after injection
x = data not obtained
T = obvious toxic symptoms; reaction time not determined.

Table 4. Increases in mean Reaction Time (sec).

Agent	Dose (um/kg sc)	No. Mice	Average Weight (g)	Time after injection (minutes) 15	30	45	60	75
norfentanyl	107	6	10 ± 3	N[a]	N	N	N	x
	214	6	10 ± 3	7	4	N	2	x
	321	8	10 ± 3	2	2	5	1	N
	43	6	20 ± 3	N	2	3	N	N
	214	6	25 ± 3	5	N	5	2	x
DNSfentanyl (XXX)	107	6	10 ± 3	N	N	N	N	x
AFII (VII)	43	6	20 ± 3	N	2	1	N	x
AFIII (VIII)	22	12	18 ± 3	3	5	4	4	N
	43	12	18 ± 3	8	14	11	7	4
	107	12	23 ± 4	N	1	1	x	x
	107	6	10 ± 3	13	18	10	6	x
α-napthoxy-FIII (XVI)	107	6	10 ± 3	N	1	3	5	x
phenoxy-FIII (XIV)	107	5	10 ± 3	N	2	N	N	x
α-napthoxy-FII.HCl	107	6	13 ± 2	N	N	N	N	x
phenoxy-FIII.HCl	107	6	13 ± 2	T[b]	1	N	6	x
AFIII-sulfonate	107	6	10 ± 3	3	N	4	x	x
	214	6	10 ± 3	N	N	N	x	x
ADIII	220	6	10 ± 3	N	5	11	6	6
ADIII-sulfonate	220	6	10 ± 3	N	3	5	5	8
anileridine	5.9 (ip)	6	10 ± 3	12	11	7	10	x
	11.8 (ip)	6	10 ± 3	22	21	22	21	x
DNS-anileridine	330 (ip)	6	10 ± 3	N	N	N	N	x
N-α-napthoic anileridine	330 (ip)	5	10 ± 3	N	5	T	T	T

N = no statistical increase from mean pre-injection reaction time of 7.5 ± 2.6 sec.

X = data not collected.

Table 5. The Effects of Fluorescent Narcotics on the Analgesic Action of Meperidine.

Agent		Dose (um/kg sc)	No. Mice	Average Weight (g)	Time After Injection 15	30	45	60	75	90
					Inhibition of Responses					
Meperidine		10	10	10 ± 3	30	13	0	0	x	x
Meperidine		20	12	10 ± 3	33	33	0	0	x	x
AFIII		107	6	10 ± 3	17	5-	5-	33	x	x
AFIII + Meperidine	AFIII	43	6	10 ± 3	67	67	67	33	x	x
	Meper.	20								
	AFIII	107	6	10 ± 3	17	33	17	0	x	x
	Meper.	10								
	AFIII	107	6	10 ± 3	67	33	17	17	x	x
	Meper.	20								
AFII + Meperidine	AFII	43	6	10 ± 3	33	17	0	0	x	x
	Meper.	10								
	AFII	86	6	10 ± 3	17	0	0	0	x	x
	Meper.	10								
Dansylfentanyl + Meperidine	DNSF	107	6	10 ± 3	0	33	33	0	x	x
	Meper.	20								
	DNSF	214	6	10 ± 3	0	0	0	0	x	x
	Meper.	20								
				ADULT MICE						
Meperidine		120	6	26 ± 2	100	100	100	83	83	50
AFII	107	107	18	24 ± 2	6	17	17	0	0	0
AFIII + Meperidine	AFIII	107	8	23 ± 4	38	63	13	25	x	x
	Meper.	20								
	AFIII	107	6	26 ± 2	100	100	100	83	33	0
	Meper.	120								
Norfentanyl + Meperidine	NF	107	6	24 ± 4	100	100	100	100	83	67
	Meper.	120								
ADIII + Meperidine	ADIII	220	6	27 ± 2	100	100	100	83	67	67
	Meper.	120								

after the injection of a threshold analgesic dose of meperidine with various doses of AFIII. As would be expected for a mixed agonist, low dose combinations of AFIII and meperidine enhance antinociceptive effect whereas higher doses of AFIII diminishes the effects of meperidine.

Dansylfentanyl and AFII demonstrate weak narcotic antagonist potential. Norfentanyl and ADIII do not possess narcotic antagonist effects. AFIII also demonstrated antagonist activity at 75 and 90 minutes after the injection of the combination of 107 uM of AFIII and 120 uM of meperidine to adult mice.

Although Albert (The Acridines p. 431) has speculated that acridine itself has narcotic antagonist activity the failure of ADIII to demonstrate such activity would indicate that the antagonist observed is not due to the acridine liberated by metabolism. This conclusion is supported by the antagonist potential of the dansylated analog of fentanyl. A new method of quantification of the analgesic activity of narcotic antagonists has recently been developed (J. Pharmacol. Exp. Ther. 192: 497, 1975).

b. ADIII and normeperidine in the brain

Studies were carried out to (1) determine the level of ADIII in the brain as a function of time, (2) determine if normeperidine is formed, (3) determine if fluorescent metabolites are formed, (4) determine if non-fluorescent metabolites are formed and where possible, their nature and amount.

Mice were decapitated at the peak of the analgesic action. To determine the presence of fluorescent moieties, the brains were homogenized with diethylamine, concentrated and then subjected to two dimensional thin layer chromatography on a 6 x 6 cm silica gel sheet (Eastman Chromagram). Numerous solvent systems were tested, the following systems proved to be the most useful: $CHCl_3$: MeOH : CH_3COOH (23:12:5, by volume) for the first dimension and $CHCl_3$: $(CH_3)_2CO$ for the second. To determine the presence of normeperidine, the brains were homogenized five passes with saline. The homogenate was sedimented at 8000 g for 30 minutes. The supernatants from both saline and acetone fractions were taken for dansylation (R.W.P. Cutler and D.S. Dudzinski: J. Neurochem. 23, 1005-9, 1974). A 5 ul of supernatant was pipetted into a capillary tube (1.6 - 1.8 x 30 mm), twice the volume of one ^3H dansylchloride (0.152 mg/ml acetone) was added, and the tube was sealed with clay. Tubes were incubated at 37°C for 30 minutes in a light tight heating plate. A 2 ul aliquot of the mixture was spotted at the corner of a 5 x 5 cm polygram, polyamide-6 TLC sheet (Brinkman Instrument, Inc.) which was developed in the first dimension with water: formic acid (100:3, v/v) and in the second dimension with n-heptane:n-butanol: acetic acid (3:3:1, by volume).

There are two fluorescent components in the brain of the ADIII injected mice; one green and the other blue. Their concentrations were too low to provide further study on quantitative and qualitative analysis by either spectrofluorometry or high

pressure liquid chromatography. Dansylnormeperidine was found in dansylated samples. Since normeperidine possessed some analgesic activity (Table 3), and convulsant properties it is not known whether the analgesic activity of ADIII is due to ADIII per se or its metabolites. However, microsomal enzyme induction by phenobarbital did not alter the onset of ADIII antinoceptive activity nor did the inhibition of normeperidine metabolism (Rodgers and Thornton, Brit. J. Pharmacol. 36: 470, 1969) potentiate the analgesic action of the analog.

c. Studies on the spectroscopy and localization of fluorescent narcotic analogs

In order to use fluorescence in research methodologies, it is necessary to have a backlog of fluorescence spectral data. That backlog should include not only the corrected excitation and emission spectra of the fluorescent narcotic analogs but also similar data on their fluorescent intermediates (and possible metabolites). In addition, it is necessary to know the spectral effects which accompany interaction (binding) of the fluorescent analogs and their intermediates with enzyme systems and model receptors. The fluorescent narcotic analogs of this study were designed to have intense fluorescence which would be readily detected by visual, microscopic or spectroscopic methods.

The ultimate objective of this approach was to provide a fluorescent analog which has known, determined binding characteristics as measured by spectral changes (quantum yield, emission maxima, or excitation changes or by energy transfer phenomena). Such a tracer molecule can then be used to determine competition for the receptor system by known narcotics and reference compounds.

This part of the pharmacological investigation was concerned with differences in the specific and nonspecific binding between selected narcotics and their fluorescent analogs and their alteration by antagonists.

The hamsters weighing about 100 grams were sacrificed by decapitation. The brains were blotted of blood and kept in ice; then homogenized five passes in 0.05 M phosphate buffer, pH 7.4 at 1.4 ml/gm wet weight. It was then centrifuged at 105,000 g for one hour at 4°C. The supernatant, the soluble fraction was divided into aliquots each for one set of experiments and frozen at 0°C until used. The protein concentration was determined by Lowry method, it was about 2.2 mg/ml. Crystallized BSA was purchased from Sigma Chemical Co., lot No. 84C-8100. It was dissolved in glass distilled water prior to the experiment. AFII and AFIII were weighed in a Cahn balance, model M-10 and dissolved in spectral grade methanol. Narcan (Naloxone-HCl, 0.4 mg/ml) was obtained from Endo Lab, Inc.

The soluble fraction was diluted 5, 10, and 20 times respectively in phosphate buffer, 0.5 ml of each was incubated with 1 ul of AFIII (9.96 mg/ml) or AFII (10.00 mg/ml) for 5 minutes at room temperature. 0.2 ml Of this incubation mixture

was then pipetted into the cuvette containing 2.3 ml of distilled water. The fluorescence measurements were made with Perkin-Elmer MPF4 spectrofluorometer.

Both AFII and AFIII have an excitation maximum at 350 nm and an emission maximum at 450 nm. In the presence of soluble protein fraction of hamster brain, the fluorescence intensity of AFIII increased from 64 to 100% which was decreased by a factor of 10% upon the addition of naloxone (naloxone: protein = $2 \times 10^{-2}/1.00$ wt/wt), indicating possible antagonism of AFIII binding. AFII fluorescence increased from 16 to 35% in the protein concentration range employed, data are given in Table 6. This may imply that a difference in their protein binding affinity contributes to the difference in their analgesic effect. AFII having little analgesic property (Table 6).

Table 6. Percent Increase in Fluorescent Intensity in the Presence of Soluble Protein Fraction of Hamster Brain

Drug	Protein Concentration 0.4 mg/ml	0.2 mg/ml	0.1 mg/ml
AFII	35 ± 15	24 ± 18	16 ± 8
AFIII	100 ± 20	76 ± 20	64 ± 15
AFIII, Naloxone	a	----	54

[a]data not obtained.

d. Binding studies

The spectrofluorometric characteristic of AFIII excitation spectra was slightly altered by the presence of the narcotic antagonist. Naloxone shifting the 270-280 nm peak into a 265 and a 280 peak (Table 7). The emission spectra of AFIII was not altered by naloxone or meperidine.

For the BSA binding studies, 2.5 ml of solution (4.76 mg/ml) was mixed with 2 ul of AFIII (50 mg/ml). Interaction at 270 was noted with a shift in the 280 peak to 295 andaccentuation ion of the 375 peak (Table). The addition of naloxone (2, 6 or 10 ul of 0.4 mg/ml) did not alter the excitation spectra.

The emission spectra of BSA and AFIII were studied using an excitation wavelength of 295 nm (tryptophan excitation). The emission maximum of AFIII was 450. BSA showed an emission maximum at 340 (tryptophan) which was reduced to 1.3 that of BSA alone, in the presence of AFIII. The E_m max of AFIII shifted to 430 in the presence of BSA. The fluorescence spectra of both BSA and AFIII were altered, suggesting binding. Addition of the narcotic antagonist to the BSA and AFIII mixture shifted that of AFIII back to 450 nm. This indicates that naloxone affected the binding between AFIII and BSA.

10. Other Pharmacological Effects - Metrazol Seizure Test

The convulsant effect of meperidine was tested by the timed continuous intravenous infusion method of Bastian (J. Pharmacol. Exp. Therap. 127: 75, 1959) as modified by Iturrian (Dev. Psychobiol. 2: 10, 1969). Mice averaged 20 ± 2 gm were injected subcutaneously with meperidine-HCl (suspended in the control vehicle) at the dose of 0.12 mM/kg 30 minutes before the intravenous infusion of 0.5% metrazol. The control mice were injected with control vehicles at 0.01 ml/gm. Meperidine has no effect on the seizure threshold (22.8 vs. 21.0 sec for the control). AFIII (107 uM/kg) and norfentanyl (321 uM/kg) were given subcutaneously 45 minutes before 0.5% metrazol infusion. AFIII injected mice did not show any seizure 60 seconds after metrazol infusion. Norfentanyl did not alter the seizure threshold. Due to vascular changes of tail veins, the intravenous injections were extremely difficult and the results variable. The bascular and anticonvulsant effects may be due to vehicle required for the fluorescent analog. Therefore, further investigations used the subcutaneous metrazol seizure test (Swinyard et al., J. Pharmacol. Exp. Ther. 106: 319, 1952) in which AFIII (107 uM/kg), norfentanyl (321 uM/kg), ADIII (140 uM/kg), normeperidine (140 uM/kg) and control vehicle (2% benzyl alcohol, 30% polyethyleneglycol 400, gs. water, .01 ml/gm body weight) were injected subcutaneously one hour before metrazol injection (85 mg/kg, sc). The results are presented in Table 8.

a. The disposition of fluorescent analogs in nontolerant and tolerant mice

Weanling mice (10 ± 3 gm) or adult (23 ± 4 gm) of the CAW: CF1 mice were used. Addiction was induced by substituting chocolate milk for the drinking water and then adding dihydromorphinone (8 mg/100 ml) to the milk for 5 days. Addiction was also achieved by intraperitoneal injection of dihydromorphine three times daily for three days at the dose of: day 1, 10.0 mg/kg/ day 2, 20.0 mg/kg and; day 3, 50 mg/kg. The degree of addiction was quantified by the method of Marshal and Winstock (1971), as over 90% demonstrated jumping behavior when injected with the narcotic antagonist naloxone. Three days after withdrawal the antinoceptive reaction time was determined.

The mice were decapitated at the end of the experiment and the brain, skeletal muscle and viscera separated and placed in iced saline solution. The brain and skeletal muscle were homogenized with diethylamine to extract the fluorescent moities and were then subjected to two dimensional thin layer chromatography (Eastman Chromagram, silica gel). For the brain extraction the first solvent system was $CHCl_3$: MeOh: CH_3COOH (23:12:5, by volume) and the second $CHCl_3$:$(CH_3)_2CO$ (1:1, v/v). For the skeletal muscle extraction, the first solvent was $CHCl_3$:MeOH:CH_3COOH (13:5:1, by volume) and the second $CHCl_3$:$(CH_3)CO$ (1:1, v/v).

Dihydromorphinone addiction produced tolerance to the analgesic effects of AFIII (Table 9).

Table 7. Spectrofluorometric study of AFIII and BSA Binding.

	Excitation Spectra (emission wavelength 450 nm)	Emission Spectra (excitation wavelength, 295 nm)
AFIII + H_2O	270-280, 350, 375	450
AFIII + H_2O + Naloxone	265, 280, 350, 375	450
AFIII + H_2O + Naloxone + Meperidine	265, 280, 350, 375	450
BSA	x	340
BSA + AFIII	295, 350, 375	340[a], 430
BSA + AFIII + Naloxone	295, 350, 375[b]	330-340, 450
BSA + Naloxone	x	340[c]

[a] 66% decrease in the 340 emission

[b] 68×10^{-6} M BSA + Naloxone-Excitation 375 increase 8%

[c] 68×10^{-6} M BSA + Naloxone-Emission 340, 13% decrease

Table 8. Metrazol Seizure Test

Drug[a]	N[b]	Time to Convulsion (seconds)	Type of Convulsion % Clonic	%Tonic	% Death
Control Vehicle	16	125 ± 31	88	45	50
Norfentanyl + v	8	87 ± 47	100	75	75
AFIII + v	8	92 ± 26	88	25	50
Normeperidine + v	8	122 ± 60	100	88	100
ADIII + v	8	95 ± 51	100	63	63
Saline	8	65 ± 14	100	100	100

[a]Dosages were given in the text; [b]number of mice used.

Table 9. Comparison of the analgesic drug action on 3 day after narcotic withdrawal and control mice.

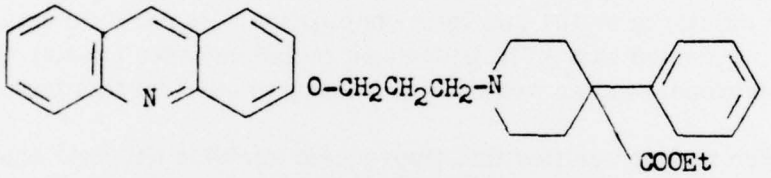
Agent	Dose (umoles/kg, sc)	No. Mice	Time after Injection (Minutes) 15	30	45	60	75
			Inhibition of Response (%)				
AFIII	107	12	8	33	17	x	x
AFIII	107 (post-addict)	12	0	17	8	x	x
AFIII + Meperidine	107 20	12	42	75	25	25	x
AFIII + Meperidine	107 20 (post-addict)	12	17	25	17	8	x

The average weight of mice is 23 ± 4 grams.

Although the injection site of sacrificed animals was extremely fluorescent, the concentration of the fluorescent moieties in the brain and skeletal muscle were extremely low. Although substantial tailing resulted from the heavy spotting of the TLC plates, one distinct blue fluorescent spot was separated by the first dimension TLC. The second dimension produced additional separation with three greenish-blue spots from the muscle extraction. The R_f values reported in Table 9 are the average of at least three experiments. No intact AFIII or 3OH acridine was detectable by this method. The most obvious alterations in the fluorescence was produced by previous naloxone administration.

Discussion

3-Hydroxyacridine, bridged by a 4 membered oxy-methylene bridge to normeperidine or norfentanyl gives fluorescent, biologically active narcotic analogs.



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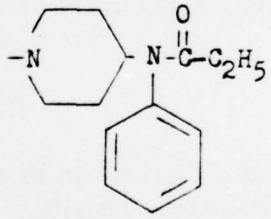

VIII

Replacement of the 3-hydroxyacridine with a-naphthol (XVI, XVII) did not eliminate analgesic activity but these analogs have less favorable spectral properties.

Compounds having a 3 membered oxy-methylene bridge were inactive. For example compounds IV and VII.

Water solubility can be increased by synthesis of analogs derived from 3-6, diaminoacridine (proflavine), for example compounds X and XI. The latter analogs are currently being studied.

As indicated above, in the alkylether bridged analogs, the 4 member bridge $-CH_2CH_2CH_2O-$, was a prerequisite for activity. The corresponding 3 membered

bridge gave inactive substrates. The fentanyl analog (AFIII-VIII) appears to be 8-10 times as active as norfentanyl and twice as active as the meperidine derivative, (ADIII-V). Norfentanyl and normeperidine are possible metabolites of AFIII (VIII) and ADIII (V) respectively. Both normeperidine and norfentanyl possess convulsive activity but normeperidine also possesses significant analgesic activity. It is reasonable that the analgesic activity of ADIII could represent a contribution from the possible metabolite, normeperidine, however, the analgesic activity of AFIII appears to be independent of any possible contribution from norfentanyl. Other possible metabolites would probably decrease the activity of AFIII, hence we believe that it is reasonable to ascribe the analgesic effect to the intact molecule.

The greater analgesic activity of AFIII in weanling mice was attributed to an approximate five-fold difference in plasma binding. A similar age dependent plasma binding for meperidine was recently reported. Meperidine metabolism and excretion is also influenced by aging.

The unexpected sharp decrease in analgesic activity as the dose of AFIII was increased from 22 um/kg to 107 um/kg in conjunction with narcotic antagonist binding studies suggested that AFIII possesses mixed narcotic agonist and antagonist properties. Additional studies would be necessary to confirm this factor.

The research to date verifies that fluorescent acridine narcotic analogs can be synthesized with a wide range of physical properties and highly useful spectral properties. The objectives of the research were met.

Conclusions

1. Fluorescent moieties can be successfully introduced into narcotic analogs with retention of biological activity.

2. Acridines are a prolific source of fluorescent moieties for incorporation into narcotic structures, particularly those derived from normeperidine and norfentanyl. A wide range of reasonable synthetic methods are available and the spectral properties of the acridine derivatives are uniformly favorable.

3. Acridines have well defined binding and energy transfer properties with proteins, enzymes and receptors which make it possible to study the effects of multiple binding sites.

4. The fluorescent narcotic analogs ADIII (V) and AFIII (VIII) have been shown to have analgesic properties related to dose and time. Data support the conclusion that the intact analogs, rather than possible metabolites, are responsible for the analgesic effect.

5. Fluorescent narcotic analogs of the acridine type can show spectroscopically the nature of binding areas and energy transfer from overlap with the emission

spectrum of protein tryptophan.

6. Fluorescent narcotic analogs can be new viable research tools.

Recommendations

The design, synthesis and pharmacological research with fluorescent narcotic analogs should be continued as new research tools for the fundamental study of narcotic systems.

Literature Cited

1. Himel, C. M. and L. M. Chan Synthesis of Fluorescent Substrate Analogs in Concepts in Biochemical Fluorescence, R. Chen and H. Edelhoch (Eds). Dekker N. Y. 1976

2. Himel, C. M., W. G. Aboul-Saad and S. Uk Fluorescent Analogs of Insecticides and Synergists J. Agr. Food Chem 19 1175 (1971)

3. Herskovits, T. T., B. Gadegbeku and H. Jaillet On the Structural Stability and Solvent Denaturation of Proteins 1. Denaturation by the Alcohols and Glycols J. Biol. Chem. 245 2588 (1970)

4. Mayer, R. T. and C. M. Himel Dynamics of Fluorescent Probe--Cholinesterase Reactions Biochemistry 11 2082 (1972)

5. Himel, C. M. and R. T. Mayer Standards for Quantum Yield of Fluorescence: 5-Dimethylaminoaphthalene-1-sulfonic Acid (Dns Acid) Anal. Chem. 42 130 (1970)

6. Ghosh, P. B. and M. W. Whitehouse 7-Chloro-4-Nitrobenzo-2-Oxa-1,3-Diazole: A New Fluorigenic Reagent for Amino Acids and other Amines. Biochem. J. 108 155 (1968)

7. Albert, A. The Acridines E. Arnold, London 1966

8. Acheson, R. M. Acridines Interscience New York 2nd Ed. 1973

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