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SYNTHESES OF ANTIBODY FLUOROCHROMES DERIVED FROM COUMARIN

by

Timothy Paul Murtha

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
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DOCTOR OF PHILOSOPHY

Major Subject: Organic Chemistry

Approved:

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In Charge of Major Work

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Ames, Iowa

1968

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INTRODUCTION

Extensive investigations have been carried out in the area of fluorescent protein labeling in the last few years. These investigations have, for the most part, used only one or two tagging reagents. Relatively little consideration has been given to the preparation and use of fluorochromes of varying colors, which could be used for the simultaneous detection of a number of antigens by means of several antibodies, each carrying its distinct fluorescent label.

This thesis is concerned with syntheses of fluorescent compounds which could be of value in the fluorescent antibody-antigen technique. These fluorochromes should prove to be chemically simpler, less expensive and of better specific fluorescence than the fluorochromes presently available.

Hydroxy and alkylaminocoumarins have been shown to be intensely fluorescent compounds. However, fluorescence intensity and wavelength of emission are dependent on the position of substitution and type of substituent on the coumarin. The objective of this thesis was the synthesis of coumarins which retain intense fluorescence upon condensation with the protein.

HISTORICAL

When foreign substances, antigens, enter an organism such as the human body, production of a specific protein called an antibody results. Antibodies are highly specific in character. An antibody will react, in most cases, only with a specific bacterium or virus. The immuno-histochemical technique takes advantage of this specificity. A specific antibody is labelled and added to a mixture of bacteria or tissue culture. Only the bacterium which causes the formation of this specific antibody should be stained. With this technique it is possible to determine rapidly whether a particular bacterium is present in a mixture of bacteria.

$$\text{ANTIBODY}_a + \text{LABEL} \longrightarrow \text{ANTIBODY}_a^*$$

$$\text{ANTIBODY}_a^* + \begin{bmatrix}\text{ANTIGEN}_a \\ \text{ANTIGEN}_b \\ \text{ANTIGEN}_c\end{bmatrix} \longrightarrow \text{ANTIBODY}_a^*\text{-ANTIGEN}_a$$

In a modified procedure, the antigenic material is isolated, purified, labelled and injected into the blood-stream. The test-animals are killed after a few days and the organ sections searched for the labelled antigenic material. Accumulation would occur where the antibody

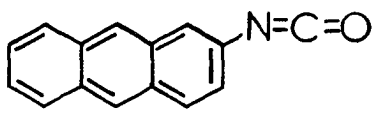
formation had taken place, resulting in identification of the organ responsible for producing each antibody. A number of papers have been published on the application of this principle and reviews are available (1, 2).

The use of labeled antibodies to detect antigen-antibody complexes in tissues is not a new idea. Initially colored dyes were used, but these were not sufficiently sensitive or specific for histochemical purposes (3). The use of radioactively labeled antibodies solved the problem of sensitivity and specificity. However, it required the preparation of highly radio-active antibodies and autoradiography for detection. The time required for detection by autoradiography may be several days and the information from the resulting autoradiograms will frequently not approach that of fluorescence microscopy (4, 5).

Fluorochromes

In order to increase the sensitivity of the dye techniques, Creech and Jones (6, 7) introduced the use of fluorescent dyes. In these early studies proteins were treated with isocyanates of aromatic polynuclear hydrocarbons to form the corresponding carbamido conjugates. Fluorochrome conjugates included 2-anthrylisocyanate (1), 1,2-benzanthryl-3-isocyanate (2a), 1,2-benzanthryl-10-

isocyanate (2b), 10-methyl-1,2-benzanthryl-3-isocyanate (2c), 1,2,5,6-dibenzanthryl-9-isocyanate (3), and 3,4-benzpyrenyl-5-isocyanate (4). Although mention was made of the strong blue fluorescence of these compounds, no further studies have been reported on actual use of these compounds



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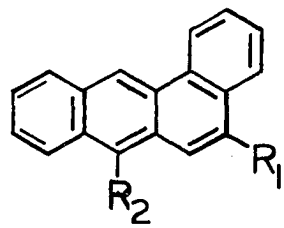
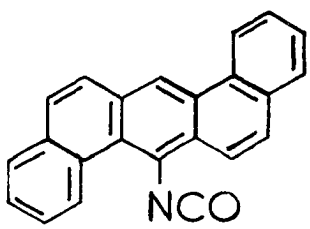


2a, R_1=NCO; R_2=H
2b, R_1=H; R_2=NCO
2c, R_1=NCO; R_2=CH_3



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as fluorescent protein tags. This may be due to poor contrast with the background autofluorescence of tissue. Autofluorescence is a natural phenomenom in biological material.

It is usually blue or green in animal and plant tissue, except for the green parts of plants, which exhibit the red fluorescence of chlorophyll. All proteins which contain one or more tryptophan or tyrosine groups show a definite fluorescence spectrum in the near ultraviolet (8, 9). The third fluorescent amino acid, phenylalanine, does not appear to contribute to the ultraviolet fluorescence of proteins. Fluorescence spectra of phenylalanine, tyrosine and tryptophan in neutral aqueous solution consist of single bands with maxima at 282, 303 and 348 mμ, respectively. However, in the protein molecule the bands shift toward the visible. Good color contrast with the normal blue-green autofluorescence may not be important if the fluorescence intensity of the tracer is sufficient. When the intensity is poor, good color contrast is essential (10).

The fluorescence of conjugates cited above inspired the labeling of proteins in a similar manner with a green fluorochrome, fluorescein isocyanate (8a) (11, 12). The preparation of 8a is shown in Figure 1. One of the complications encountered in this synthesis is the formation of two nitro isomers (5) and (6) which are very difficult to separate. Separation can be effected by fractional crystallization of the nitro acetates (13) or more efficiently by chromatography (14).

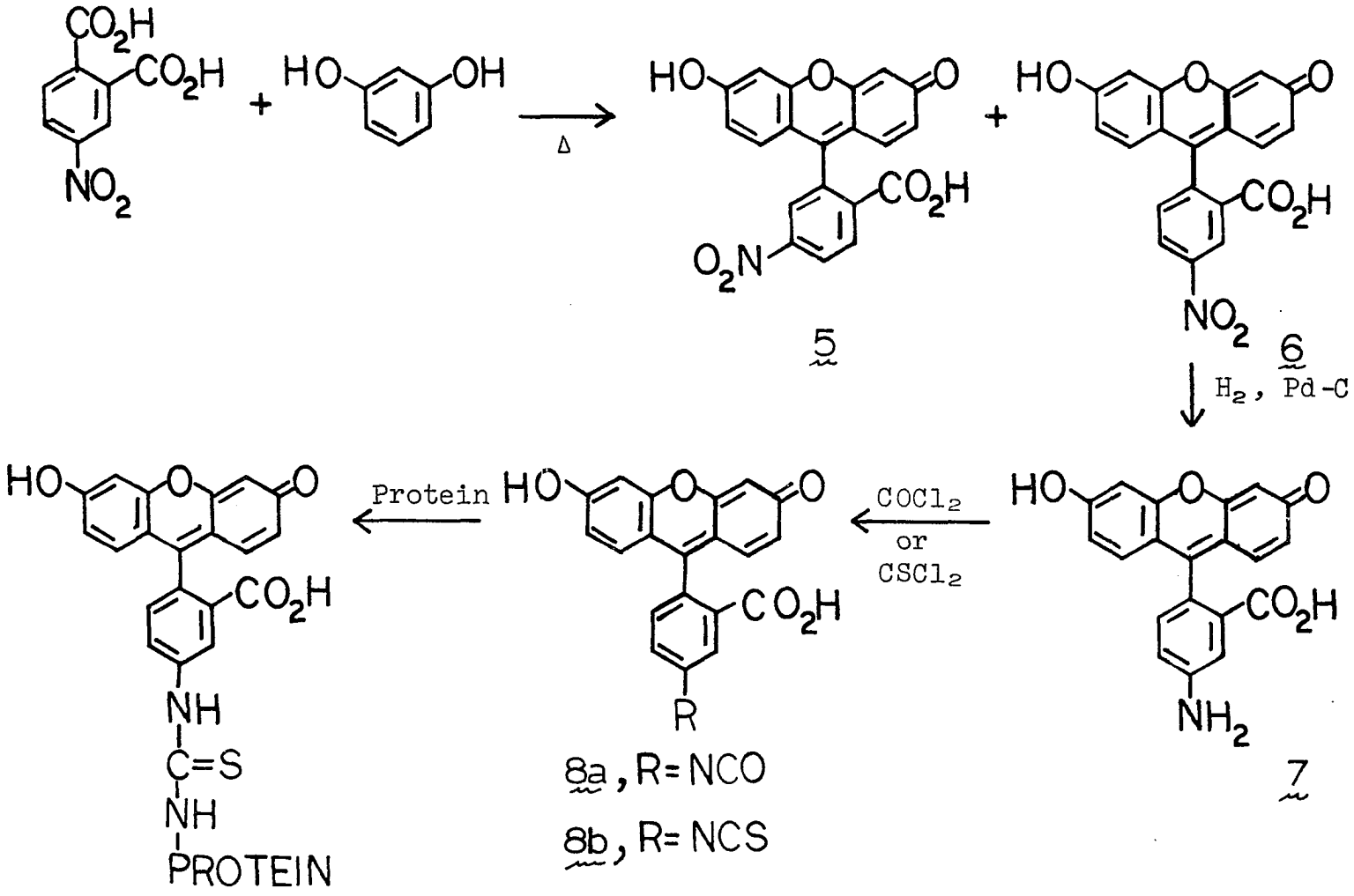
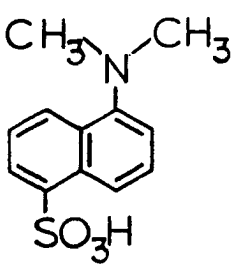


Figure 1. Synthesis and conjugation of fluorescein isothiocyanate.

Aminofluorescein (7) is obtained from nitrofluorescein (6) by catalytic hydrogenation and converted to the isocyanate with phosgene. Unfortunately, the isocyanate is sensitive to moisture and conjugation must be carried out soon after preparation. Fluorescein isocyanate does not provide good color contrast to tissue; however, the intensity of fluorescence is strong enough to counteract this drawback in most cases.

Fluorescent conjugates have also been prepared from 1-dimethylaminonaphthalene-5-sulphonic acid (15) (DANS) (9) and have been used successfully for tracing by Clayton (16) and Mayersback (17). However, the fluorescent color of DANS is very similar to fluorescein isocyanate and diminished considerably in intensity. Clayton also used a diazo derivative of nuclear fast red (benzaldehyde-6-nitro-2-sodium diazotate) (10) to obtain red fluorescent conjugates, but apparently no extension of this work has been done.



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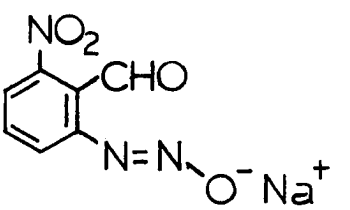


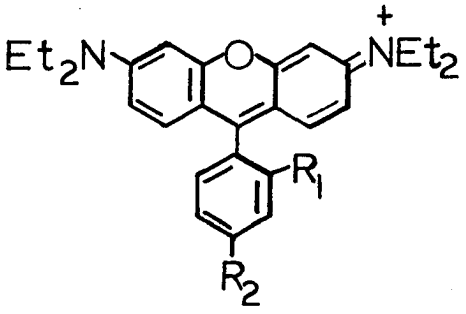
10

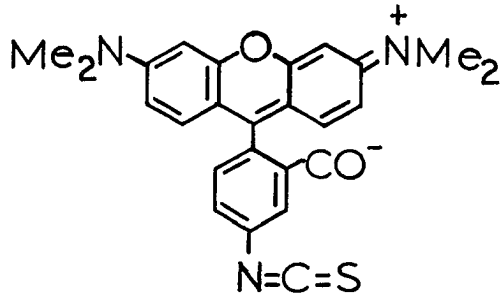
In order to obtain a conjugate of contrasting color to fluorescein isocyanate, Silverstein (18) introduced the orange-fluorescent compound, rhodamine B isocyanate [3,6-tetraethyldiamino-9-(2'-carboxy-4'-isocyanato)xanthene] (11a). He found that satisfactory labeling to protein was difficult to reproduce (10).

The introduction of the isothiocyanate functional group into fluorochromes has greatly simplified protein conjugation (19). Fluorescein isothiocyanate (8b) is prepared by treating aminofluorescein with thiophosgene instead of phosgene. This procedure has the advantage of using a less toxic reagent and the stability of the product is considerably better. However, it fails to overcome the disadvantage of poor color contrast to tissue autofluorescence. Similarly, the isothiocyanate of rhodamine B has superseded the isocyanate (19).

Hiramato (20) introduced tetramethylrhodamine isothiocyanate (12) which is comparable in staining power to the corresponding tetraethyl derivative. Fluorescence of 12, like 11b, is not intense and its principal application is for staining of antigen tissues with blue or green autofluorescence.

Chadwick and coworkers (21) investigated several alternatives to fluorescein isothiocyanate. Among these,





IIa, $R_1=CO_2^-$; $R_2=NCO$

IIb, $R_1=CO_2^-$; $R_2=NCS$

IIc, $R_1=SO_3^-$; $R_2=SO_2Cl$

IId, $R_1=H$; $R_2=NCO$

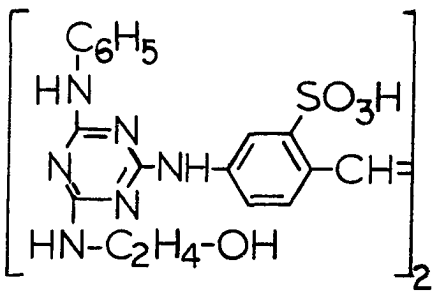
IIe, $R_1=H$; $R_2=NCS$

IIf, $R_1=H$; $R_2=N_2^+Cl$

12

lissamine rhodamine B [3,6-tetraethyl-9-(2'-sulfo-4'-sulfonylchlorophenyl)xanthene] (IIc) was found to be suitable because of its orange color and the intensity of the fluorescence. The other alternatives included fluolite C {4,4'-bis[2-anilino-4-(2-hydroxyethylamino)-1,3,5-triazine-6-ylamino]-2,2'-stilbenedisulfonic acid} (13), lissamine flavine FFS [p-(2,4-dinitroanilinophenylazo)benzenesulfonic acid] (14), R4388 (2H-naphtho[1,2-d]triazol-2-yl-2-stilbenesulfonic acid) (15), lissamine rhodamine GS [2,7-dimethyl-3,6-diethyl-9-(2'-sulfo-4'-sulfonylchlorophenyl)xanthene] (16), amino-eosin [2,4,5,7-tetrabromo-3,6-dihydroxy-9-(2'-carboxy-4'-isocyanato)xanthene] (17), 3-phenyl-7-isocyanatocoumarin (18),

and 5-β-carboxyethylaminoacridine (19). Although all showed strong fluorescence when unconjugated, the intensity is lowered on conjugation and their weak blue fluorescence contrasts poorly with tissue autofluorescence. Thus, value as a fluorochrome is diminished.



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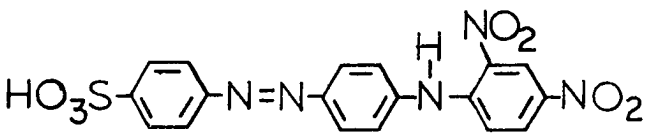

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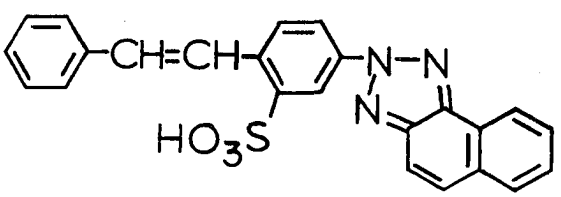

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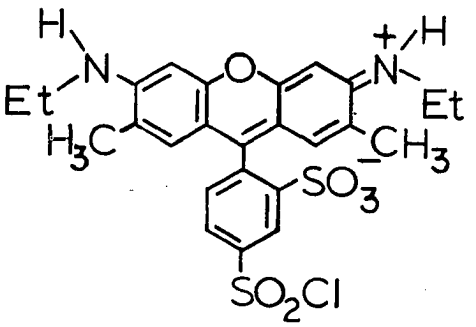

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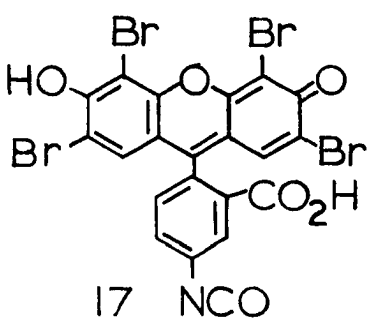

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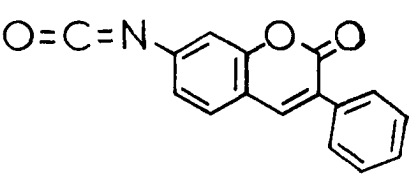

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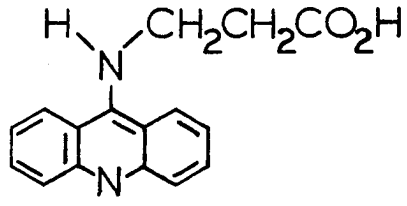

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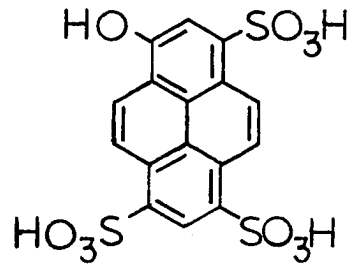

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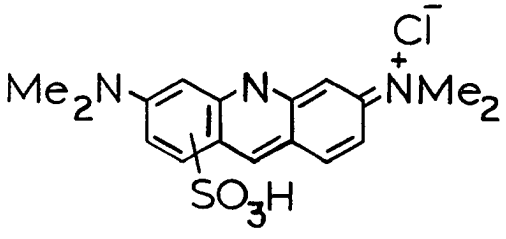

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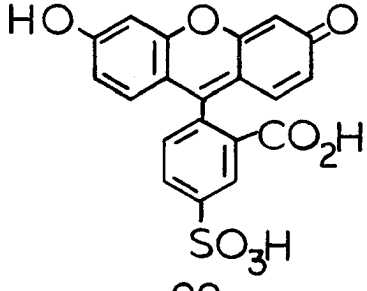


22

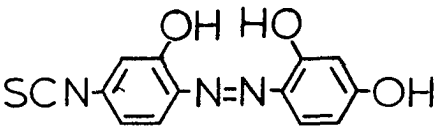
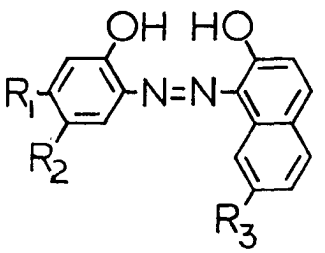
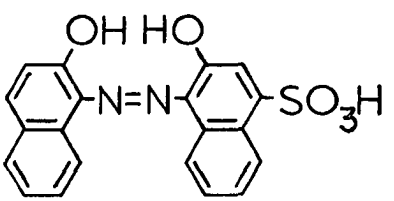
The sulfonylchloro group has been used in the conjugation of a large number of fluorescent dyes. In addition to compounds 13, 14, 15 and 16, Uehleke (22) conjugated 3-hydroxypyrene-5,8,10-trisulfonic acid (20), sulfoacridine orange (21), lissamine rhodamine B (11c), and sulfofluorescein [3,6-dihydroxy-9-(2'-carboxy-4'-sulfonic acid)xanthene]

(22). Only the rhodamine derivative has proved of any practical importance. Sulfofluorescein shows the green fluorescence resembling that of fluorescein isothiocyanate, but with reduced intensity. 3-Hydroxypyrene-5,8,10-trisulfonic acid has a maximum emission very similar to that of fluorescein isothiocyanate but there are too many reactive groups in the molecule which cause cross-linking (23).

Borek and Silverstein (24) prepared aminorosamine [3,6-tetraethyl-9-(p-aminophenyl)xanthene] and conjugated it to protein by isocyanate (11d) isothiocyanate (11e) and a diazonium salt (11f). The diazo method proved to be the better method, but the fluorescence intensity of the conjugate is weak at neutral pH and strong at pH 3.0. This seriously limits their usefulness because antigen-antibody interaction is greatly reduced at pH 3.0 (10). Like all the other available orange fluorescent dyes, aminorosamine shows fluorescence of a lower order of intensity than fluorescein.

In a slightly different method Dowdle and Hansen (25) conjugated 2,2', 4-trihydroxy-4-isothiocyanatoazobenzene (23) to protein. These compounds are not fluorescent themselves but, after chelation with an aluminum ion, show yellow fluorescence (26). The chelation has been carried out at pH 5.2 and the aluminum complex, when once formed, is quite stable and is not broken when the pH is changed to slight alkalinity. These complexes may thus be used together

Table 1. Spectra data of Al-chelated azo dyes

Fluorochrome	Excitation (mμ)	Emission (mμ)	pH	Ref.
				
23	285,365,492	565	5.2	23
				
24, R_1=NH_2; R_2,R_3=H	267,375,535	582	5.2	23
25, R_1=NH_2; R_2=H; R_3=OH	267,390,535	588	5.2	23
26, R_1=H; R_2=Cl; R_3=H	250,383,535	612	5.2	23
				
27	365,535-553	605	5.2	23

with other fluorochromes which are quenched at acidic pH.

When the size of the dihydroxyazobenzene molecule is increased by addition of further benzene rings, the excitation bands, as well as the emission, move towards the longer wave length

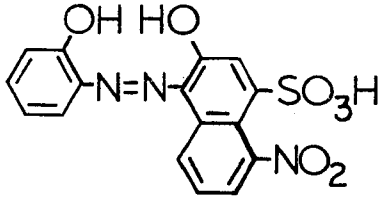
as shown by 1-(2-hydroxy-4-aminophenylazo)-2-naphthol (24), 1-(2-hydroxy-4-aminophenylazo)-2,7-dihydroxynaphthalene (25), 1-(2-hydroxy-5-chlorophenylazo)-2-naphthol (26), and 1-(2-hydroxy-4-sulfonaphythylazo)-2-naphthol (27) in Table 1 (23).

One of the most serious difficulties to overcome in fluorescent antibody technique is non-specific staining, i.e., fluorescent staining of microscopic preparations not due to specific reaction between a particular antigen and its corresponding labeled antibody. This may be caused by free label not bound stoichiometrically to protein or by the combination of properly labeled antibodies with tissue cells because of polar groups active on the label. While the first can be eliminated by gel filtration, the latter presents serious difficulties. At pH 7.0 the antibody conjugates have a negative charge and will stain positively charged tissue proteins. Since removal of unwanted label is difficult, methods to prevent this problem have been attempted (10, 19).

Introduction of a counterstain along with the desired label has reduced this problem. Smith and co-workers (27) treated sections with mixtures of rhodamine-B-conjugated non-immune antibody and fluorescein-conjugated immune antibody to obtain specific green fluorescent staining against an orange background. It was found that the procedure masks the blue autofluorescence and inhibits non-specific staining

by the immune conjugate.

Recently (28, 29, 30) azo-dyes were screened for possible use as fluorescent counterstains to mask auto- and non-specific fluorescence. The dyes were pontachrome blue black (27), flazo orange (26), eriochrome black [1-(2-hydroxyphenylazo)-5-nitro-4-sulfo-2-naphthol] (28), acid alizarin garnet (2,2',-4-trihydroxy-5-sulfoazobenzene) (29) and diamond red (30).



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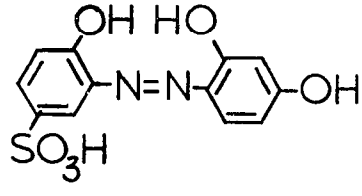

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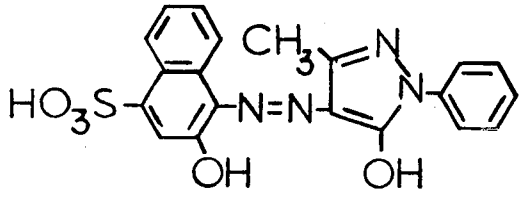

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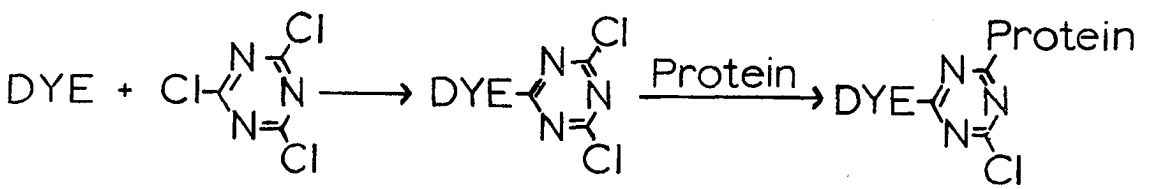
Of the five azo-dyes evaluated, three were satisfactory as counterstains , flazo orange, pontochrome blue black and eriochrome black. Flazo orange masked all auto- and non-specific fluorescence. It also was the most highly fluorescent of the three and gave the best uniform contrasting background to fluorescein. Diamond red and acid alizarin garnet were ruled out as counterstains on the basis of the poor contrast to fluorescein isothiocyanate and their inability to

mask non-specific fluorescence.

The reaction of dyes containing amino groups with cyanuric chloride to produce "reactive dyes" has been advocated by Hess and Pearse (31). Chadwich and Narin (32) found that proteins labeled by this method with proflavine (2,8-diaminoacridine) and rheonine [2-amino-8-dimethylamino-5-(p-dimethylaminophenyl)acridine] lost their fluorescence on conjugation and their histological staining power was almost negligible.



Of the various fluorochromes prepared and tried, only three have proved satisfactory for routine use as fluorescent protein tracers (10). The three tracers are lissamine rhodamine B (11c), fluorescein isothiocyanate (8b) and DANS (9) and of these three, fluorescine is by far the most popular because of its color and intensity of fluorescence.

Hansen (23) suggested that the ideal compound for labeling proteins should have the following properties:

1. It should be a compound which can be prepared of high purity and be reasonably stable.

2. It should not have more than one reactive group to avoid cross-linkage.

3. Polar groups should be absent to avoid non-specific adsorption because of the charge on the label.

4. Maximum fluorescence at pH range at which immunological reactions are carried out (pH 7-10).

5. Conjugation with globulin should not diminish the intensity of the fluorescence.

6. The fluorescent antibody should absorb the energy emitted by the mercury lines.

7. The excitation spectrum should be well separated from the fluorescence spectrum.

8. The conjugated dye should be resistant to radiation and not show fading.

No labeling material is known which fulfills all of the requirements. Fluorescein isothiocyanate meets requirements 1, 2 and 4 well, but not the remainder.

Reactive Sites of Proteins

Several different chemical groups are available in protein for attachment of fluorochromes. In addition to the free amino and carboxyl groups at the ends of each protein chain, there are terminating imidazoles, aliphatic hydroxy, phenolic, ϵ-amino and sulfhydryl groups (5, 10).

Determining the actual site of conjugation by hydrolyzing

the conjugate and determining the amino acid to which the fluorochrome is attached has not been carried out on fluorescent conjugates. Studies in which simple molecules with a reactive group, such as sulfonyl chloride, are combined with protein provide some indication of the site of reaction. Gurin and Clarke (33) hydrolyzed benzenesulfonylated gelatin and showed that the sulfonyl chloride reacts with the ϵ-amino groups of the lysine residues. Phenylisocyanate and isocyanates of polynuclear hydrocarbons have shown indication of conjugation with the lysine residues (7, 34). Diazonium salts have been shown to couple with the free amino group (35) and to the phenolic moiety of tyrosine and histidine (36).

By analogy with the above it appears that the ϵ-amino group of lysine is the most probable site of conjugation of lissamine rhodamine B and fluorescein isothiocyanate.

Fluorescence of Fluorochromes

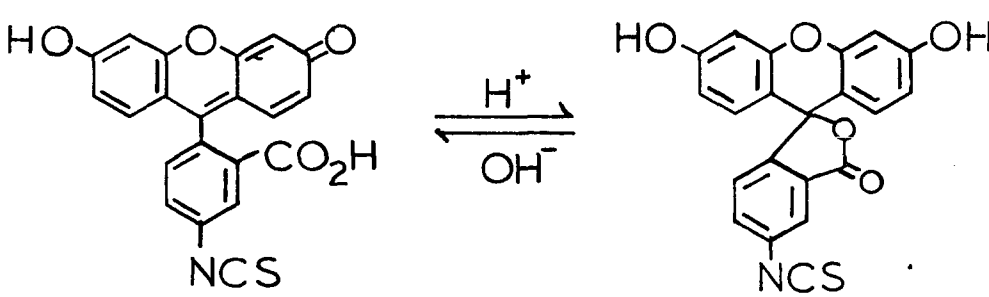
Although considerable interest has been displayed in fluorescent protein tracing, accurate data on absorption and emission are frequently omitted. A comparison of one dye with another is usually based on subjective impressions. A knowledge of excitation and fluorescence data for each dye is necessary for choice of filters and source of radiant energy. Unfortunately, convenient sources of short wave radiation are few and it has not been possible to select one which gives

maximum intensity at the wavelengths which are preferentially absorbed by the fluorochrome (23). The mercury arc, which is most widely used in fluorescence microscopy, has an uneven spectrum in which the following lines are of most importance: 313, 334, 365, 404, 435, 546 and 577 mμ. The strongest emission lines are at 365 and 435 mμ (10).

Table 1 and Table 2 summarize the spectra data which are available for the known fluorochromes. Since labels only have practical importance if they are excited at the several wavelengths emitted by the mercury arc, fluorochromes with absorption maxima at 365 and 435 mμ are most desirable.

Fluorescein isothiocyanate has been used more than all the other labels together. Although fluorescein isothiocyanate has many good qualities, it meets requirements 1, 2 and 4 cited by Hansen (23) very well, it is deficient in many respects. Its absorption curve correlates poorly with the mercury arc spectrum. Its fluorescence intensity is strongest when excited at wavelengths near 490 mμ. However, the 490 mμ peak is too close to the corresponding emission peak of 520 mμ, to permit satisfactory selective filtering. A filter which will stop such primary illumination will also reduce the transmission of fluorescence (10). Therefore, in fluorescent protein labeling of fluorescein isothiocyanate the absorption maximum at 360 mμ is used for excitation. This results in a loss of fluorescence intensity due to a

lower molar extinction coefficient at this wavelength. A further loss is encountered when the fluorochrome is bound to the protein. Decreases of 90% and 82% in fluorescence intensity have been reported (37, 38). This discrepancy may be due, at least partly, to difference of the pH during measurement. In the fluorescein conjugate it was found that fluorescence intensity increased with pH to 10.7 where the intensity was found to be twice that at pH 7.0 (39). In the free dye maximum fluorescence occurred at pH 8.7 where the intensity was about 10% greater than that at pH 7.0. Below pH 7.0 fluorescence is inhibited due to conversion of 8b to 8c (40). In an acid solution the carboxyl group forms a cyclic "lactoid" ring which prevents the tautomeric shift and thus inhibits fluorescence. This ring is cleaved in alkaline solution, resonance is enhanced, and fluorescence is intensified.



8b 8c

Table 2. Spectra data of fluorescent conjugates

Fluorochrome	Excitation (mμ)	Emission (mμ)	pH	Ref.
Anthracene (1)	350,365,385	400		5
1,2,5,6-Dibenzanthracene(3)	327,342,357,377,399	390,415,450		5
Fluorescein[a]	494	518		41
	495	527		3
Fluorescein isocyanate (8a)	495	520		13
	495	550		41
Fluorescein isothiocyanate (8b)	280,320,490	517	7.6	23
Dimethylaminonaphthalene-5-sulfonic acid chloride (9)	243,255,330	528	7.0	10,23
Rhodamine B isothiocyanate (11b)	300,350	595		24
Lissamine Rhodamine B (11c)	257,280,352,568	597	8.0	23,24
Rosamine B isothiocyanate (11e)	255,563	582	8.0	23
	300	625	3.0	24
Tetramethylrhodamine isothiocyanate (12)	257,281,356,555	580	8.0	23

[a]Basic ring system with no function groups attached.

Table 2 continued

Fluorochrome	Excitation (mμ)	Emission (mμ)	pH	Ref.
3-Hydroxypyrene-5,8,10-tri-sulfonic acid chloride (20)	242,285,374,400	515	7.0	23
	235,290,452	515	8.0	23
Fluolite C (13)		blue[b]		21
Lissamine flavine FFS (14)		blue-green[b]		21
R4388 (15)		dull-green[b]		21
Lissamine rhodamine GS (16)		orange-yellow[b]		21
Aminoeosin (17)		yellow[b]		21
3-Phenyl-7-isocyanatocoumarin (18)		blue[b]		21
5-Carboxyethylaminoacridine (19)		blue-green[b]		21

[b]No spectra data was reported, therefore, only the color of fluorescence is indicated.

Lissamine Rhodamine B (11c) has become the most frequently used labeling material after fluorescein isothiocyanate, because of the orange-red fluorescence. Its absorption maximum at 352 mμ is well excited by the 365 mμ mercury line. The absorption maximum at 568 mμ is not suitable, since it is too close to the emission maximum at 597 mμ. The fluorescence intensity is much lower than that found for fluorescein isothiocyanate making it less desirable. There is also a change in the color of fluorescence from red to orange on exposure to intense ultraviolet radiation. This suggests that photochemical decomposition of the conjugate is taking place, since the color of fluorescence reverts to that found for the free dye (10, 23).

Fluorescence of lissamine rhodamine B is almost independent of pH but its conjugates give different intensities at various pH values. The fluorescence emission of conjugates of lissamine rhodamine B show little variation near neutrality, but it is approximately doubled at pH 4.0 and pH 10.5 (10).

Tetramethylrhodamine isothiocyanate absorbs well at 365 mμ, but the intensity of the emission at 580 mμ is low. Rosamine isothiocyanate has no advantage over the labels just mentioned and is seldom used.

Dimethylaminonaphthalene sulfonic acid has a very intense absorption at 243 and 255 mμ with a weaker absorption at 330 mμ. Since the mercury arc emission falls off sharply below

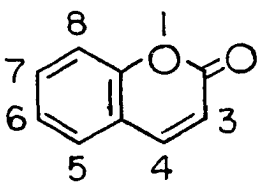
300 mμ, the lower maxima cannot be utilized. The absorption of the mercury lines at 334 mμ produces fluorescence color very similar to that of fluorescein isothiocyanate but with reduced intensity. Furthermore, on irradiation in ultraviolet light, the fluorescence fades and releases the label from the protein (10, 23).

3-Hydroxypyrene-5,8,10-trisulfonic acid has a broad absorption band at 374 mμ, a band at 400 mμ at pH 7.0, and a broad band at 451 mμ at pH 8.0. These match the emission spectrum of the mercury arc very well. The emission (515 mμ) is very similar to fluorescein isothiocyanate but of reduced intensity. The compound has an added disadvantage that there are too many active groups which cause cross-linking in conjugation (23).

The 2,2′-dihydroxyazobenzene dyes (Table 1) have fluorescence spectra which make them useful in fluorescence microscopy (23). They have the advantage that the maxima for excitation and emission are well separated and broad enough to match the mercury emission lines. They also fluoresce in the long wavelength region which may make them useful in double tracing experiments. They have the disadvantage of being pH sensitive and of low fluorescence intensity.

Chemistry of Coumarins

Fusion of a pyrone ring with a benzene nucleus gives rise to a class of heterocyclic compounds known as benzo-pyrones. Two types are of interest, benzo-α-pyrones (coumarins) (31) and benzo-γ-pyrones (chromones) (32).



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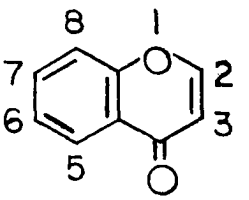

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A large number of derivatives are known for both ring systems. Several serve a useful purpose as sedatives, flavor extracts, perfumes and as a blood anti-coagulent. Coumarin (31), the parent substance of the benzo-α-pyrone group, was first discovered in the tonka bean in 1820 and later synthesized by Perkin (42, 43).

The preparation and chemistry of the synthetic coumarins has been extensively reviewed (44, 45). More recently, the naturally occurring coumarins have been reviewed (46).

Coumarins can be synthesized from a variety of starting materials. The choice of starting material depends on whether substitution is desired in the aromatic ring or in the pyrone ring. Of the procedures known the von Pechmann condensation is the most useful (47). It proceeds from very

simple starting materials and gives good yields of coumarins substituted in the pyrone ring. The yields of coumarin is dependent on the substituents in the phenol, on the type of β-keto ester, and on the condensing agent used.

Condensation of ethyl acetoacetate (33) with phenol in sulfuric acid gives only a trace amount of 4-methylcoumarin (34) (Figure 2). Substitution of electron-donating groups in the meta position of phenol allows condensation to occur more readily. Groups such as hydroxyl, methoxyl, amino, alkylamino, dialkylamino and alkyl in the meta position of phenol provide good yields of coumarins by this process (48, 49). Electron-donating groups in the ortho or para position of phenol seem to afford a substrate with the same reactivity of phenol itself. Electron-attracting groups, when present in the phenol, inhibit the condensation (49).

In addition to acetoacetic ester, various substituted acetoacetic esters enter into the condensation. α-Alkyl (50) and α-arylacetoacetic esters (51, 52) have been reported to give coumarins. With less reactive phenols, α-substituted β-keto esters give decreasing yields of the resulting coumarin (53). In substituted alkyl acetoacetic esters such as ethyl α-cyanoacetoacetate (54) or ethyl α-acetylaceto-acetate (55), elimination of the α-substituted group occurs in the condensation.

A number of reagents can be used for the von Pechmann condensation (45). The most common reagents for condensation

with resorcinol or alkylresorcinols are concentrated sulfuric acid (47), hydrogen chloride in acetic acid (56) or in alcohol (57), and polyphosphoric acid (58). Zinc chloride is the best reagent for aminophenols (59). Phosphorus pentoxide has also been used in the condensation, but it has the disadvantage of causing formation of chromones in select cases (45).

There are two possible routes of reaction between a β-keto ester and a phenol under these conditions. Route A gives rise to a coumarin derivative (34) and route B gives rise to a chromone derivative (35) (Figure 2).

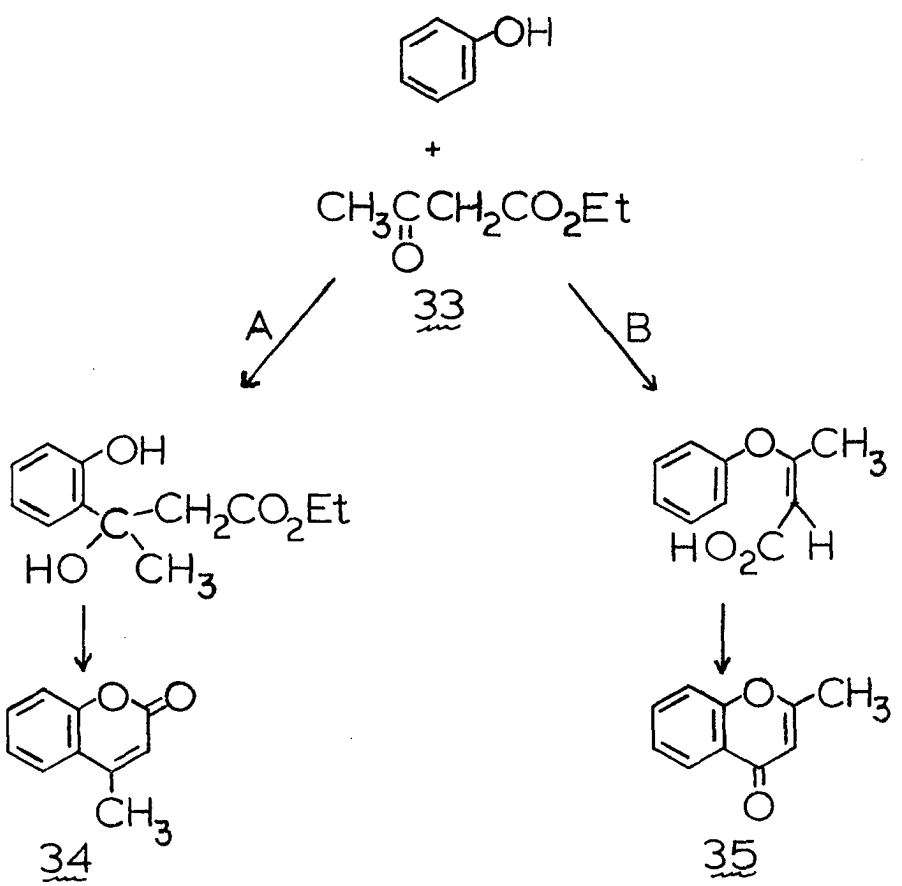


Figure 2. Reaction routes between a β-keto ester and phenol.

Simonis claimed that condensation of β-keto esters with phenols in the presence of phosphorus pentoxide gives chromones instead of coumarins (60, 61, 62). However, a number of workers have shown that this is not the case (63, 64, 65). In general, the following points can be made:

1. When sulfuric acid serves as a condensing agent, a coumarin derivative is always obtained.

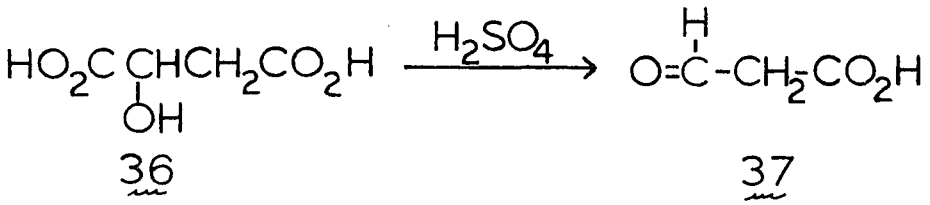
2. Phenols which react readily in the presence of sulfuric acid (resorcinol, pyrogallol, orcinol, α-naphthol) also give coumarins in the presence of phosphorus pentoxide.

3. Phenols which do not form coumarins at all or form them in poor yields with sulfuric acid, give chromones by the Simonis reaction in the presence of phosphorus pentoxide.

4. Phosphorus pentoxide is only condensing agent which usually favors chromone formation.

Coumarins without substituents in the pyrone ring can be prepared from malic acid (36) and phenol (Figure 3). Malic acid is decomposed by concentrated sulfuric acid to carboxyacetaldehyde (37) which then condenses with the appropriate phenol (45).

Coumarins can also be prepared from salicylaldehydes by the classical method of Perkin (42, 43) (Figure 4). A major disadvantage of these procedures is unavailability of the appropriate salicylaldehyde and low yields in the subsequent reaction.



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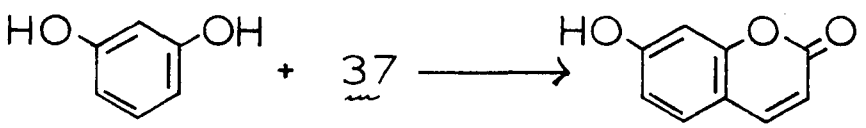
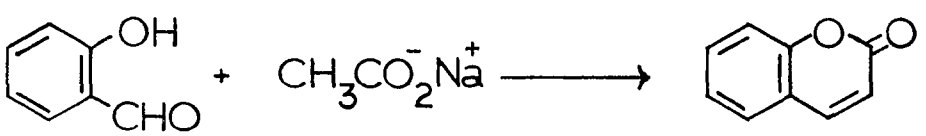


Figure 3. Synthesis of coumarins unsubstituted in the pyrone ring.



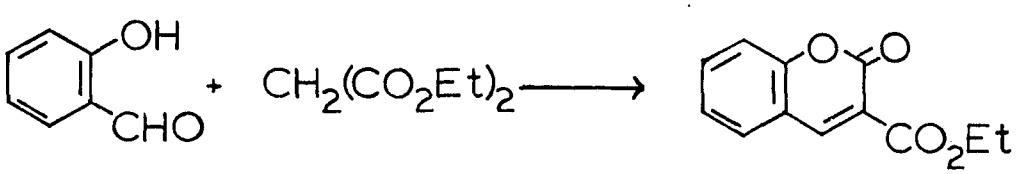
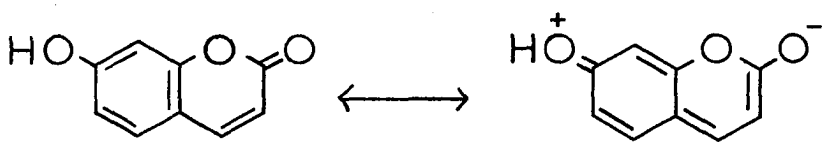


Figure 4. Syntheses of coumarins by the Perkin condensation.

Fluorescence of Coumarins

Early studies on the fluorescence intensities of variously substituted coumarins can only be evaluated in a qualitative manner. The first studies (66, 67, 68) used sunlight to excite fluorescence and the results were evaluated visually. The low excitation energy and the sensitivity of the eye make these results questionable. It was reported here and in subsequent studies (69, 70, 71) that the presence and position of the hydroxyl group is a deciding factor in the fluorescent property of coumarins. Strongest fluorescence is observed with the 7-hydroxy derivative (38) (70). Coumarins with hydroxyl in the 3-, 4-, 5- and 8-positions were found to be non-fluorescent. The 6-hydroxy derivatives show a weak blue-green emission in acid, but not in alkali or alcohol. It was postulated that added resonance associated with the pyrone ring was responsible for increased fluorescence (72). This could explain why the 6- and 8-hydroxy derivatives were non-fluorescent, since this type of



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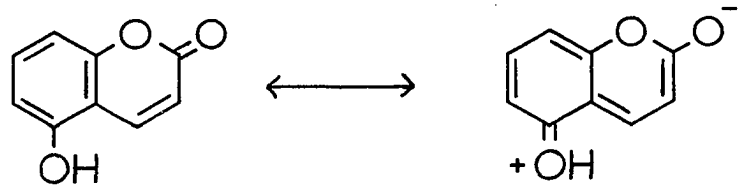
resonance cannot take place. However, it is not clear why 5-hydroxycoumarin (39) is less fluorescent than 6-hydroxycoumarin since resonance into the pyrone ring can occur.



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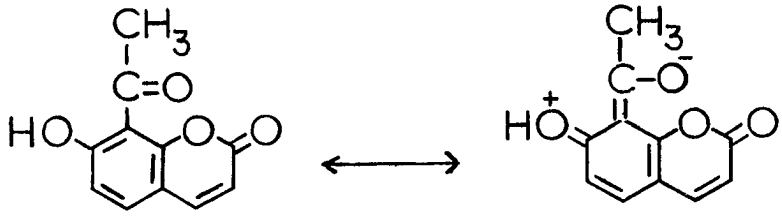
Introduction of a second hydroxyl at the 5- or 6-position of 7-hydroxycoumarin causes a fifteen-fold reduction in maximum fluorescence intensity. Substitution of hydroxyl, methoxyl, acetyl or acetoxyl at the 8-position of 7-hydroxycoumarin quenches all fluorescence (70). The non-fluorescence of the 8-acetyl-7-hydroxycoumarin (40) is postulated to be caused by inhibition of the normal movement of charge from the hydroxyl to the pyrone ring (72).



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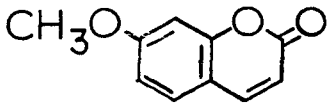
The methoxyl is much less effective than the hydroxyl group in influencing the fluorescence of the molecule. 7-Hydroxycoumarin is 300 times as fluorescent as 7-methoxycoumarin (41) and 5,7-dihydroxycoumarin (42) is 200 times as fluorescent as 5,7-dimethoxycoumarin (43). The lower

fluorescence efficiency may be due to a lower mesomeric effect of a methoxyl compared with that of a hydroxyl group (72).



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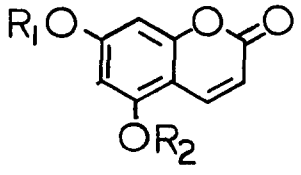


42, R_1R_2=H

43, R_1R_2=CH_3

Acetylation of the hydroxyl group causes the fluorescence intensity to decrease, but these products are still twice as fluorescent as the corresponding methoxy derivatives (72).

Substitution at the 3-position with electron-attracting groups increases fluorescence intensity, while similar substitution at the 4-position quenches fluorescence (67, 70, 72). An increase in the fluorescence intensity compared to 7-hydroxycoumarin is noted for the following 3-substituted derivatives: 3-carboxy-, 3-carboxamido-, 3-phenyl-, 3-acetyl, 3-carboethoxy- and 3-cyano-7-hydroxycoumarin (72). Only 3-benzoyl-7-hydroxycoumarin showed a decrease in fluorescence intensity (67, 72).

It has been shown that coumarins with electron repelling groups in the 4-, 6- or 7-position or electron attracting groups in the 3-position cause a shift of fluorescence to longer wavelength. When the substitution occupies the

7-position of 4-methylcoumarin, the shift of fluorescence to longer wavelengths is greatest with the diethylamino group, less with the hydroxyl group and least with the methoxy group (73).

Recently Sherman and Robins (74) found what appears to be a correlation of fluorescence intensity and the Hammett substituent constant. The fluorescence intensity of 3-substituted 7-hydroxycoumarins was plotted against Hammet σ values for different types of substituent interactions. The data correlate best with the Hammett σ values obtained for meta substituted benzoic acids. No explanation was given to explain why the meta σ values should correlate if the 3-substituted coumarins are simply vinylogs of a para-substituted phenol.

RESULTS AND DISCUSSION

Syntheses of 6 and 8 Substituted 7-Hydroxy-4-methylcoumarins

7-Hydroxy- and 7-diethylaminocoumarins are of theoretical importance because they are strongly fluorescent molecules. They are of practical interest as potential fluorochromes for protein labeling. Since no procedures have appeared in the literature for the preparation of 7-hydroxy-4-methylcoumarin isothiocyanates or 7-diethylamino-4-methylcoumarin isothiocyanates, it was necessary to explore synthetic pathways to these compounds.

The advantages of substituted 7-hydroxy and 7-diethylaminocoumarins as potential fluorochromes over those known at present are ease of preparation, high yields, maximum fluorescence at pH range at which immunological reactions are carried out (pH 7-10), an excitation spectrum, well separated from fluorescence spectrum, and fluorescence excited by light of wavelength 365 mμ which is the principal line of the mercury spectrum.

Since no fluorescence data has been reported on the amino or isothiocyanatocoumarin derivatives, it was necessary to prepare a series of substituted coumarins in order to determine the most highly fluorescent derivative. It has been shown that both hydroxyl and amino groups increase the intensity of fluorescence of many aromatic compounds (75). Therefore, it was expected that the amino derivatives would

parallel the existing data on the hydroxyl derivatives. However, it is not always possible to predict which position of substitution will give the maximum fluorescence intensity.

Introduction of a second hydroxy at the 6- or 8-position of 7-hydroxy-4-methylcoumarin reduces the fluorescence intensity considerably and a similar decrease would be expected for the corresponding amino derivative. However, the isothiocyanate group should show considerable difference in fluorescence intensity due the increased π-electron system and to lower basicity of the nitrogen. Conjugation of the isothiocyanate with the protein results in a thiourea linkage (Figure 1) which suggests that fluorescence of the fluorochrome should resemble the intensity of the corresponding acetamide. In support of this postulate it has been reported (76) that 8-acetamido-7-hydroxy-4-methylcoumarin (54) showed intense fluorescence in basic solution while the 8-amino-7-hydroxy-4-methylcoumarin (47) did not fluoresce. However, no comparison was given with 7-hydroxy-4-methylcoumarin to indicate whether the intensity had increased or decreased.

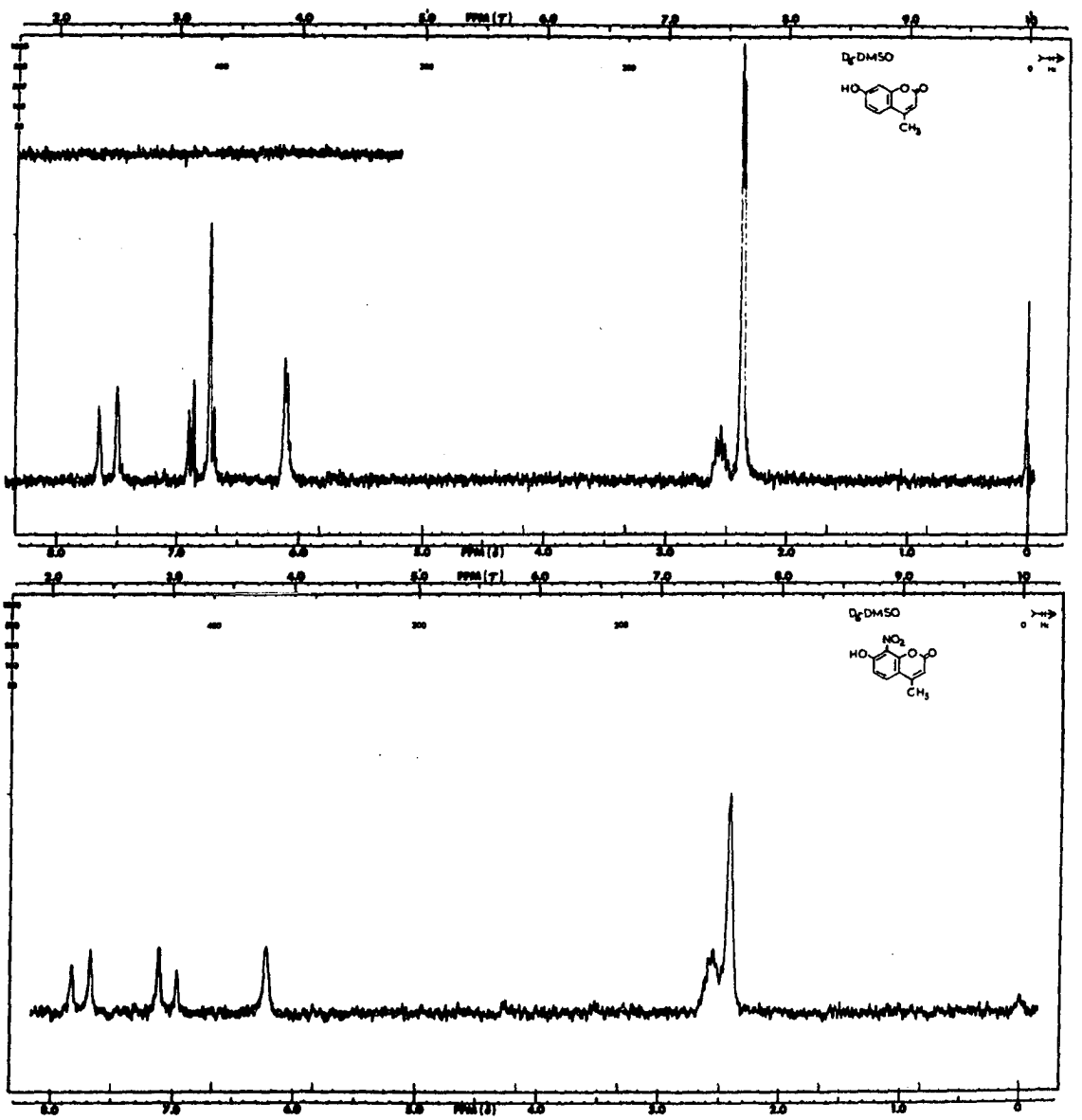
The preparation of what appeared to be the simplest possible member of the series was desirable, namely the 6- or 8-isothiocyanato derivative. Since electrophilic substitution on 7-hydroxy-4-methylcoumarin would be expected at the 3-, 6- or 8-position, it is of interest to know how the nmr spectrum will change for each substitution.

The nmr spectrum of 7-hydroxy-4-methylcoumarin is shown in Figure 5. The doublet at 2.38 ppm was assigned to the methyl group which shows fine allylic coupling to the olefinic proton at 6.1 ppm (J = 1.0 Hz). The protons in the aromatic region showed an ABX pattern in which $J_{BX} \cong 0$. The C_5 proton appeared as a doublet (centered at 7.58 ppm) which was coupled to the C_6 proton (J = 9 Hz), but was not coupled to the C_8 proton. The C_6 proton (6.8 ppm) was coupled to both the C_8 proton (J = 1.5 Hz) and the C_5 proton (J = 9 Hz). The C_8 proton resonance is superimposed upon the C_6 proton at 6.7 ppm (J = 1.5 Hz).

Substitution on the aromatic ring of 7-hydroxy-4-methylcoumarin should simplify the aromatic region of the nmr spectrum. If substitution occurs at the 8-position, the aromatic region should show an AB pattern with an ortho coupling constant of 7-10 Hz. Substitution at the 6-position should show an AX pattern in the aromatic region. The coupling constant should be small (1 Hz) due to the para position of the protons. Substitution at the 3-position would result in loss of the olefinic proton and subsequent loss of the allylic coupling to the C_4 methyl group. Therefore, it should be a simple procedure to determine at which position the substituent had entered.

Nitration of 7-hydroxy-4-methylcoumarin (44) gave two isomers (Figure 6). The 8-isomer (45) was chosen for study, since it is produced in better yield and is easier to purify

Figure 5. Nuclear magnetic resonance spectra.
a: 7-Hydroxy-4-methylcoumarin (44).
b: 7-Hydroxy-4-methyl-8-nitrocoumarin (45).


D6DMSO
HO
CH3
D6DMSO
NO2
HO
CH3

than the corresponding 6-nitro isomer (46). The nmr spectrum of 45 is shown in Figure 5. As expected the aromatic region is simplified. An AB pattern was shown at 7.35 ppm (J = 9 Hz). The olefinic proton (6.2 ppm) and the C_4 methyl (2.4 ppm) showed little change in position from 44. The large coupling constant in the AB pattern of the aromatic region confirmed the position of substitution.

Reduction of 45 to 8-amino-7-hydroxy-4-methylcoumarin (47) was effected with sodium hydrosulfite in concentrated ammonia (76). Treatment of 47 with thiophosgene did not give the expected isothiocyanate (48), but produced a compound (49) which appears to be a 2-benzoxazolinethione derivative.

The structure was supported by analytical, (Experimental) and spectra data. The ir spectra showed N-H stretching bands (3440, 3180, 3150 and 3080 cm^{-1}) characteristic of a thioamide and the hydrogen bonded tautomer (24). The ir spectra also showed carbonyl (1755 and 1730 cm^{-1}) and double bond (1650 and 1600 cm^{-1}) stretching frequencies which are typical of the α-pyrone system. The mass spectrum showed molecular ion (m/e 233) and an (M + 2) ion which is 6% of the molecular ion. This indicates that there is one sulfur atom in the molecule.

To further ascertain the structure, it has been shown that 2-benzoxazolinethione (51) can be prepared from o-aminophenol (50) and thiophosgene in the presence of pyridine (77).

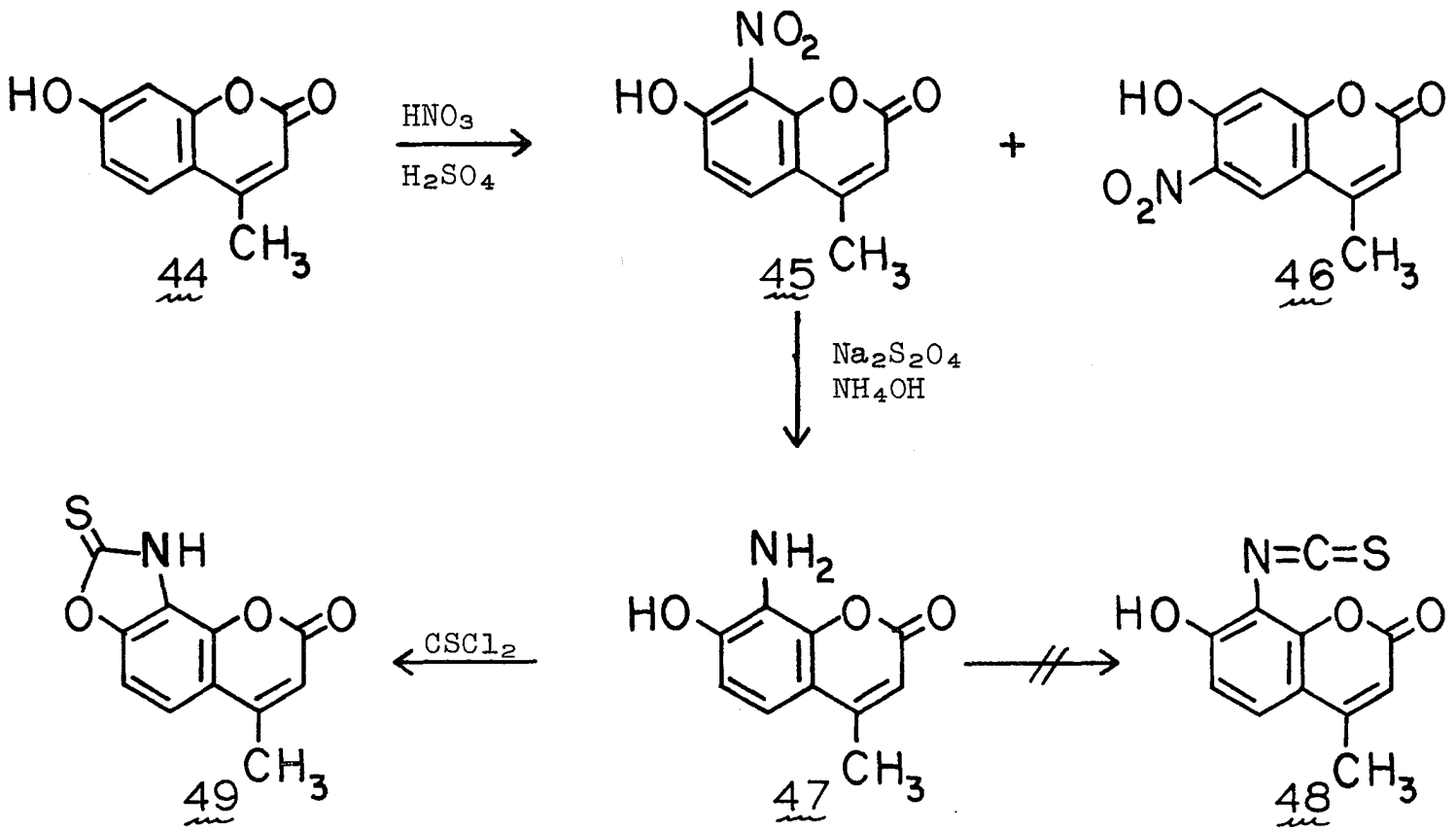
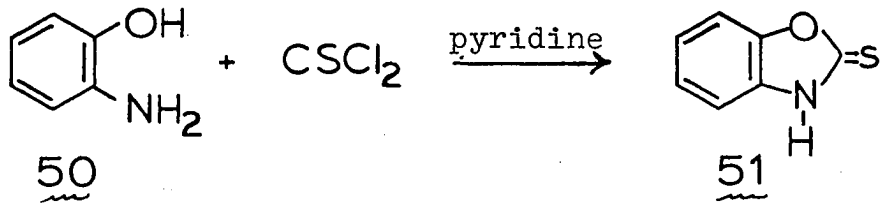


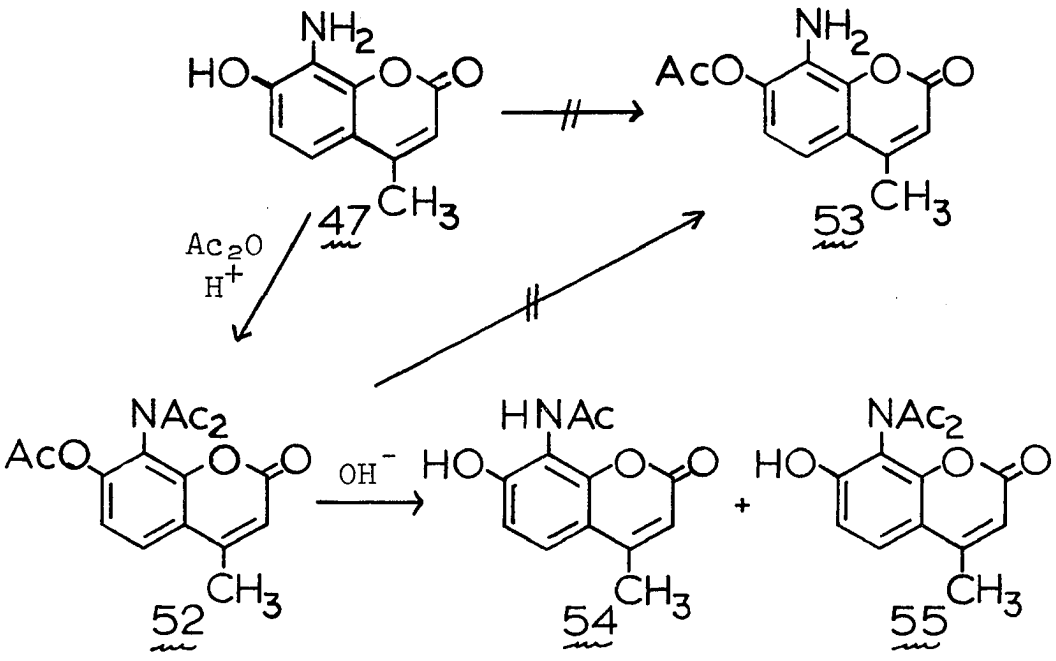
Figure 6. Attempted synthesis of 7-hydroxy-8-isothiocyanato-4-methylcoumarin (48).

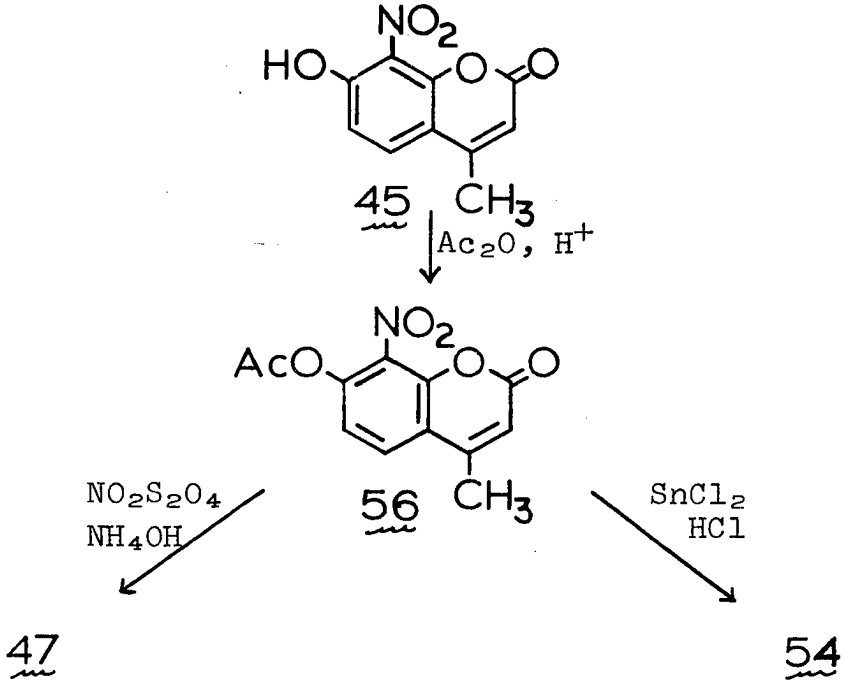


50 51

In order to obtain the 8-isothiocyanato derivative (48) it appeared necessary to protect the phenol, make the isothiocyanate and then remove the protecting group either before or after conjugation with the protein. Since the isothiocyanate group is fairly stable under acidic conditions, it was postulated that an acetate could be used as a protecting group and subsequently removed without destroying the isothiocyanate. However, when 47 was acetylated with acetic anhydride and a few drops of sulfuric acid only the triacetyl derivative (52) was obtained. A similar problem was encountered when acetylation was carried out under basic conditions (78). Careful hydrolysis of the triacetyl derivative gave 8-acetamido-7-hydroxy-4-methylcoumarin (54) and 8—N,N-diacetamido-7-hydroxy-4-methylcoumarin (55). None of the desired 7-acetoxy-8-amino-4-methylcoumarin (53) was obtained (78).

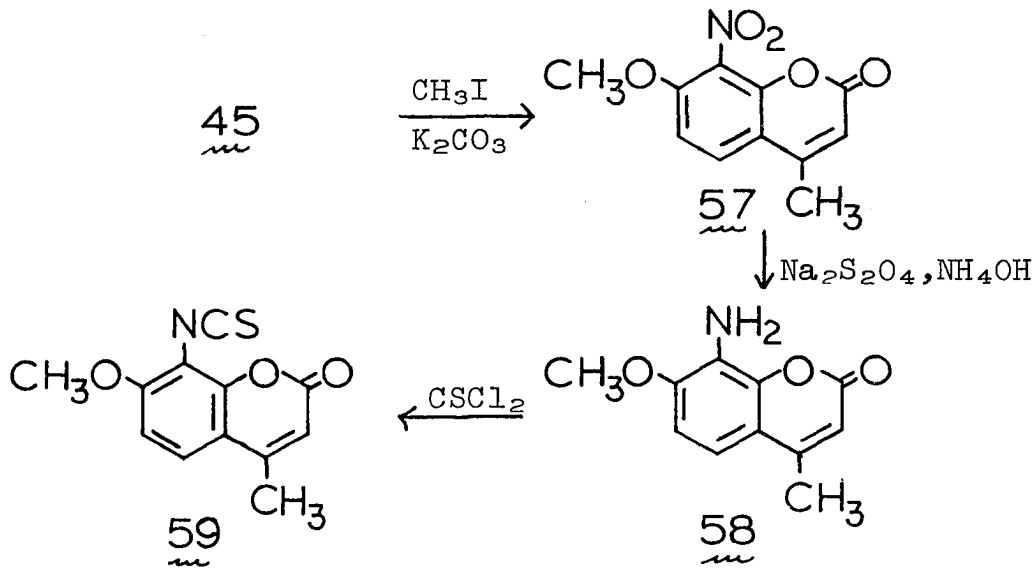
In a slightly modified procedure 45 was acetylated with acetic anhydride to give 7-acetoxy-4-methyl-8-nitrocoumarin (56). Reduction of 56 under basic conditions did not give 53. The strenuous conditions reduced the nitro group and,


NH2
HO
O
O
CH3
47
AcO
53
Ac2O
H+
NAc2
52
OH−
HNAc
54
+
55


NO2
HO
45
CH3
Ac2O, H+
AcO
56
NO2S2O4
NH4OH
SnCl2
HCl
47
54

at the same time, cleaved the acetate to give 8-amino-7-hydroxy-4-methylcoumarin (47). Reduction under acidic conditions resulted in the isolation of 54 rather than the desired O-acetyl derivative 53 (78).

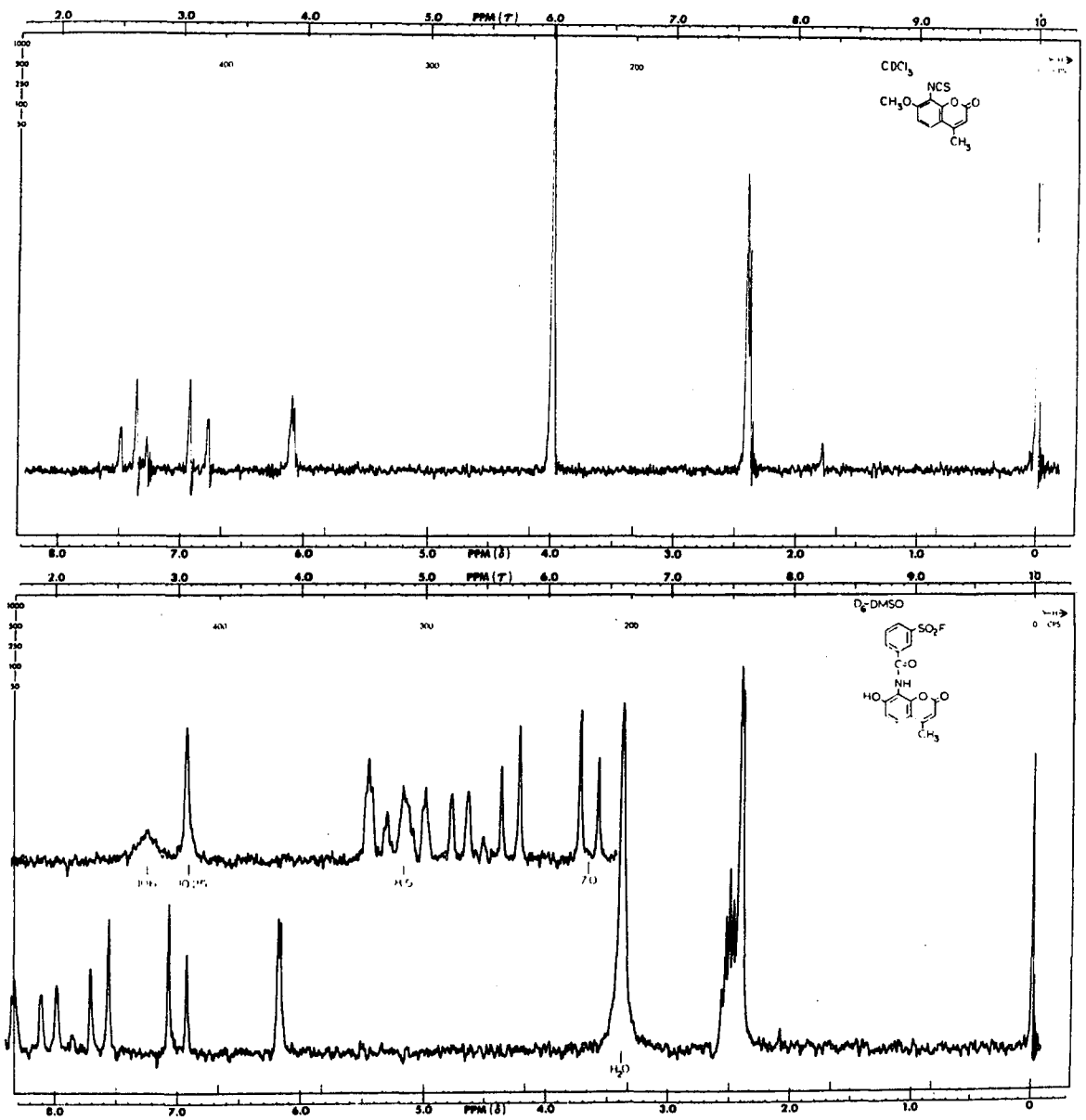
In view of the experimental difficulties encountered in the synthesis of 7-acetoxy-8-amino-4-methylcoumarin, protection of the phenol with an ether group was attractive. 7-Methoxy-4-methyl-8-nitrocoumarin (57) can be prepared in good yields from 45 upon treatment with methyl iodide and potassium carbonate. Reduction of the nitro derivative (57) to the amine (58) can be effected with stannous chloride and hydrochloric acid (78) or with sodium hydrosulfite. 8-Amino-7-methoxy-4-methylcoumarin was easily converted to the isothiocyanate (59) on treatment with thiophosgene in acetone. The ir spectrum of 59 showed a strong isothiocyanate band (2050 cm^{-1}), characteristic of an aromatic isothiocyanate. Strong bands were observed at 1740, 1610 and 1570 cm^{-1} characteristic of the α-pyrone system. The nmr spectrum (Figure 7) is typical of 4,8-disubstituted 7-hydroxycoumarins. The doublet (2.4 ppm) is assigned to the C-4 methyl group. Fine allyic coupling to the olefinic proton was observed at 6.1 ppm. The 3-proton singlet (4.0 ppm) is assigned to the 7-methoxyl group. The two aromatic protons appear as an AB pattern centered at 7.15 ppm (J = 9 Hz). The fluorescence spectrum showed a maximum at 410 mμ when excited



at 350 mμ. However, the fluorescence intensity is less than 1% of that of 7-hydroxy-4-methylcoumarin. This reduction in intensity may be due to either the substitution at the 8-position or the methyl ether group at the 7-position. Since it has been reported that the fluorescence intensity of 7-methoxy-4-methylcoumarin is diminished by a factor of 300 in the conversion from 7-hydroxy-4-methylcoumarin (70), no conclusion can be made as to whether the 8-isothiocyanate is quenching or not. It appears that a free phenol is required in order to obtain maximum fluorescence. However, attempts to cleave the 7-methyl ether with acid resulted in hydrolysis of the isothiocyanate.

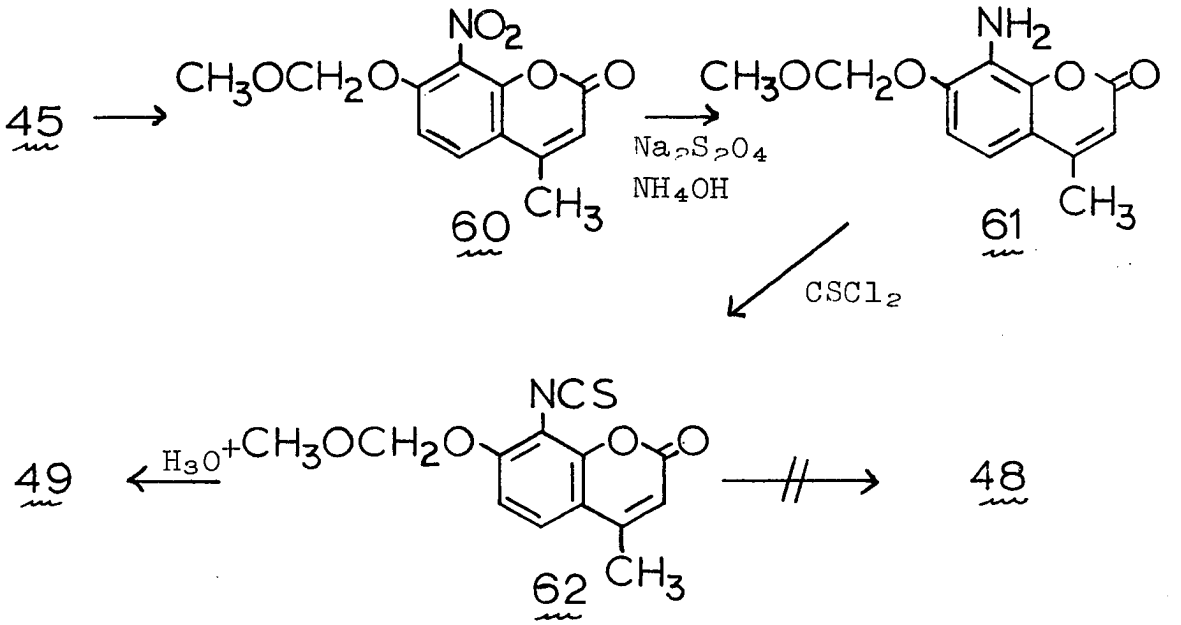
It is well known that acetals can be cleaved readily and under very mild acidic conditions. This prompted the

Figure 7. Nuclear magnetic resonance spectra.
a: 8-Isothiocyanato-7-methoxy-4-methylcoumarin (59).
b: 8-[m-(Fluorosulfonyl)benzamido]-7-hydroxy-4-methylcoumarin (66).


PPM (τ)
PPM (δ)
CDCl3
NCS
CH3O
CH3
D6-DMSO
SO2F
C=O
NH
HO
H2O

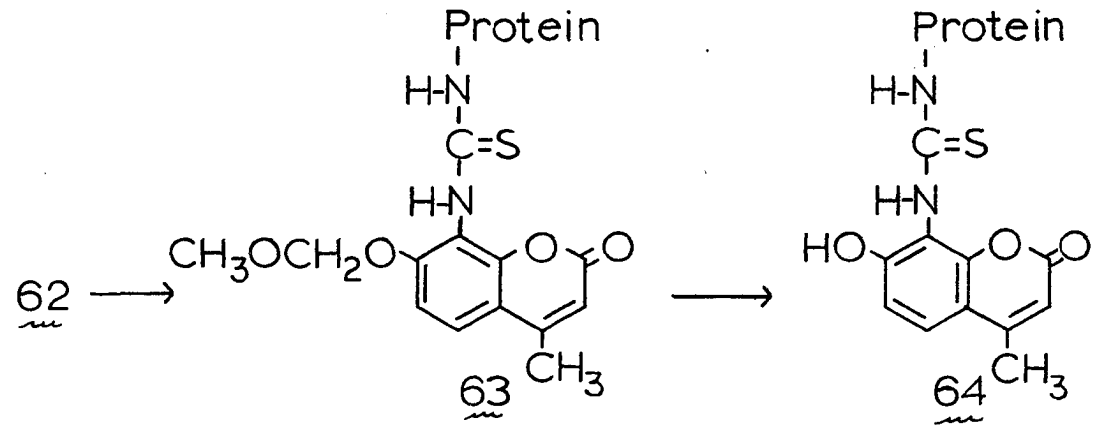
investigation of a phenolic acetal as a protecting group. Attempts to obtain the tetrahydropyranyl ethers of 45 and 47 with dihydropyran were unsuccessful (79). This may be attributed to strong hydrogen-bonding of the phenol to the ortho substituent. Since preparation of the methyl ether (57) was accomplished readily, the preparation of the methoxymethyl ether was expected to occur with the same ease. Indeed, when 45 was treated with chloromethoxymethane (chloromethyl methyl ether) and potassium carbonate in acetone the methoxymethyl ether (60) was obtained in 93% yields. Conversion to the amine (61) in 83% yield was effected with sodium hydrosulfite in ammonia. Condensation of the amine with thiophosgene in acetone was unsuccessful. Only starting material (61) was isolated. When the reaction was carried out with thiophosgene in water, good yields of 8-isothiocyanato-7-methoxymethyl-4-methylumbelliferone (62) were obtained. Apparently water solvates the reaction site better than acetone.

The ir spectrum of 62 showed a strong isothiocyanate absorption band (2050 cm^{-1}) and strong absorption bands at 1740 and 1610 cm^{-1} which are characteristic of the α-pyrone system. The nmr spectrum of 62 showed singlets at 5.33 and 3.54 ppm which were assigned to the methylenedioxy and methoxy, respectively. The olefinic proton (6.18 ppm) and the AB pattern (centered at 7.25 ppm, J = 9 Hz) in the aromatic region are characteristic of the

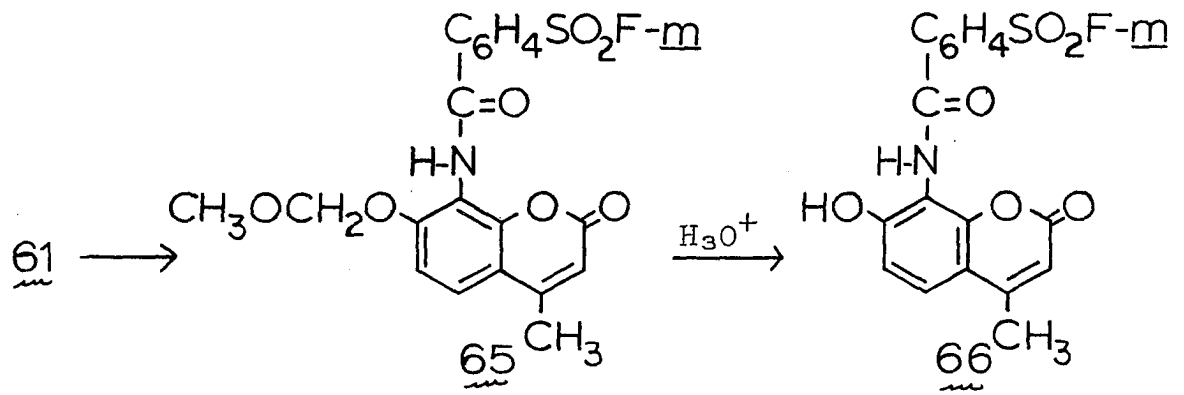


8-substituted 7-hydroxy-4-methylcoumarin. When 62 was irradiated with a mineral lamp the observable fluorescence was comparable to 59. Cleavage of the acetal (62 → 48) with dilute mineral acid at room temperature was not successful, resulting in isolation of starting material. When higher temperatures were applied only the 2-benzoxazolinethione derivative (49) was obtained. Conjugation of 62 with a protein (62 → 64) would eliminate the problem of intramolecular condensation, but the conditions required for the acetal cleavage are most surely too severe for the protein to remain intact.

Since 8-acetamido-7-hydroxy-4-methylcoumarin (54) is strongly fluorescent, it would seem that an 8-benzamido

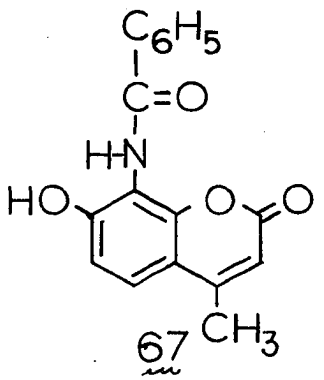


derivative should be equally fluorescent. A functional group which is stable to acid hydrolysis attached to benzoyl chloride would be a convenient one-step synthesis of a fluorochrome from 61. The sulfonylchloro group has been used in a number of previous cases for conjugation with protein. This suggested the use of fluorosulfonyl group as another method of conjugation. The fluorosulfonyl group is remarkable stable in neutral or acid media and hydrolyzed easily with alkali (80). These properties were used to advantage in the synthesis of 8-[m-(fluorosulfonyl)benzamido]-7-hydroxy-4-methylcoumarin (66). 8-Amino-7-methoxymethyl-4-methylumbelliferone (61) was converted to the amide (65) with m-(fluorosulfonyl)benzoyl chloride. Hydrolysis of the acetal with acid gave a 75% yield of 8-[m-(fluorosulfonyl)benzamido]-7-hydroxy-4-methylcoumarin (66). The ir spectrum of 66 showed strong carbonyl bands (1700 and 1665 cm^{-1}) as well as O-H and N-H stretching bands (3315 and 3260 cm^{-1}). The nmr spectrum

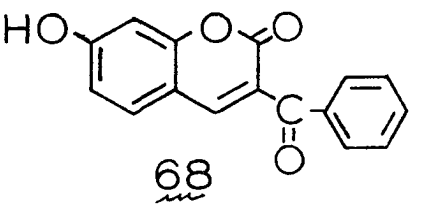


(Figure 7) showed, in addition to the AB pattern of an 8-substituted 7-hydroxy-4-methylcoumarin, a low field 4-proton multiplet centered at 8.34 ppm which was identical in shape and position to the nmr spectrum of m-(fluorosulfonyl)benzoyl chloride. In addition, a broad 1-proton peak (10.6 ppm) and a 1-proton singlet (10.25 ppm) were assigned to the phenolic and amide protons, both of which disappeared on adding D_2O. The fluorescence spectrum of 66 showed a maximum at 395 mμ in methanol and 460 mμ in aqueous basic solution (pH 10). However, the intensity is very low in both solvents, less than 1% of that observed for 7-hydroxy-4-methylcoumarin. Since the 8-acetamido derivative (54) is 100 times more fluorescent than 66, it was thought that the fluorosulfonyl group may be the cause of quenching. However, when the corresponding unsubstituted 8-benzamido-7-hydroxy-4-methylcoumarin (67) was examined, it was found to be nearly identical in fluorescent intensity to 66. It appears that the benzoyl

group is the cause of quenching. A similar quenching effect was noted for 3-benzoyl-7-hydroxycoumarin (68) (74).



67



68

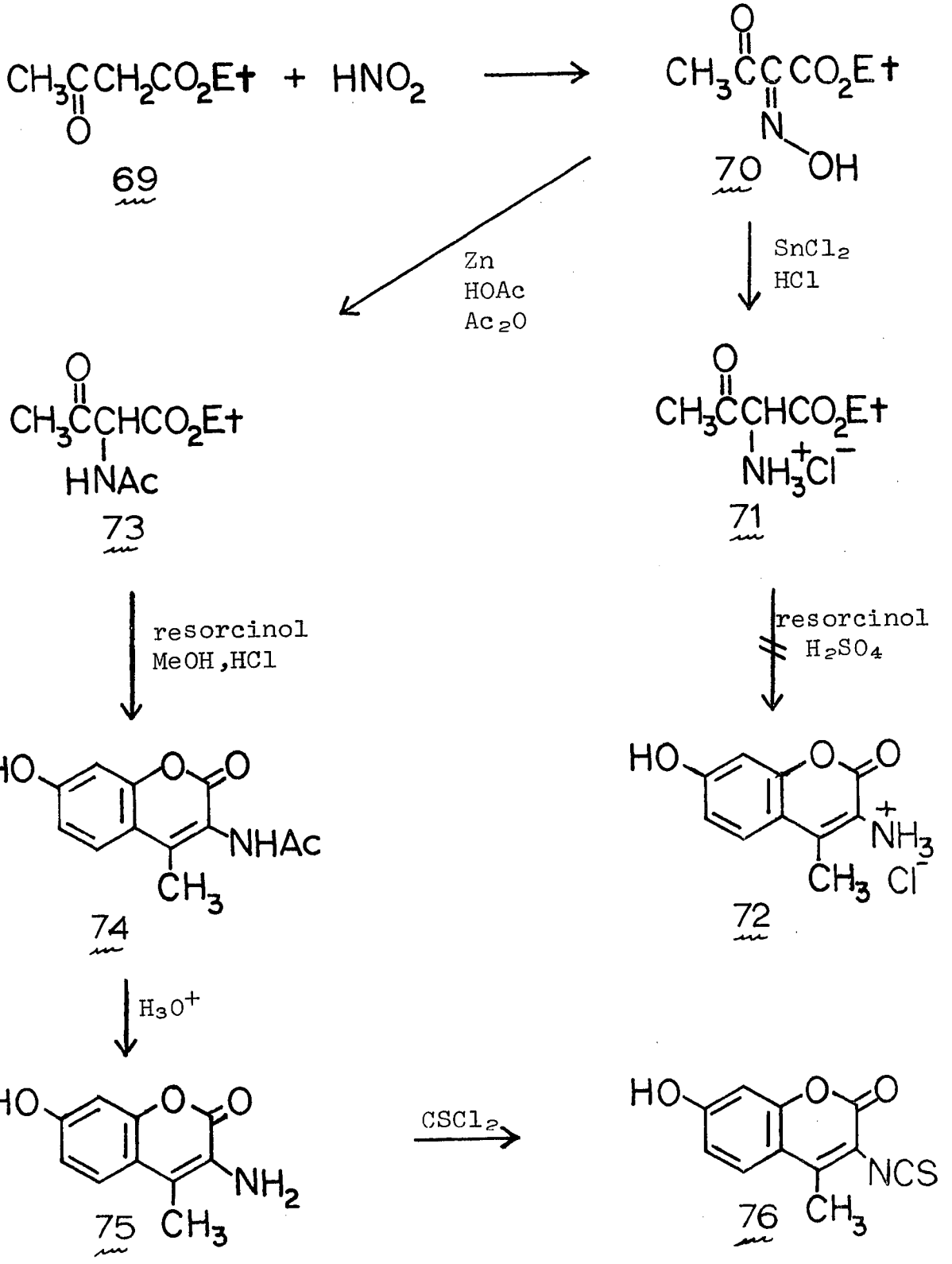
Syntheses of 3-Substituted 7-Hydroxy-4-methylcoumarins

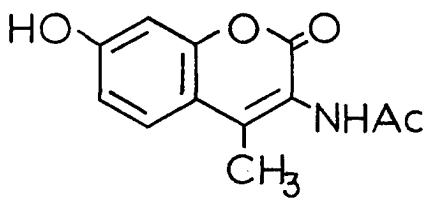
Since fluorescence is strongly dependent on substitution on the coumarin nucleus and is very sensitive to quenching from aromatic substitution, an isothiocyanate substituted on the pyrone ring was considered. Direct substitution at the 3-position with a nitro or amino group is not feasible and therefore the appropriately substituted acetoacetic esters must be prepared and subsequently condensed with resorcinol. The sequence (69 → 72) (Figure 8) was postulated as a method for making 3-amino-7-hydroxy-4-methylcoumarin hydrochloride (72). Nitrosation of ethyl acetoacetate (69) by the method of Meyer (81) as modified by Albertson, et al, (82) gave crystalline ethyl α-oximinoacetoacetate (70). Reduction of of the latter with stannous chloride in concentrated hydrochloric acid gave ethyl α-aminoacetoacetate hydrochloride

(71) (83, 84). The free amino derivative is unstable and condenses with itself. When 71 was condensed with resorcinol in sulfuric acid or refluxed with zinc chloride in ethanol none of the expected product (72) was obtained. The uv spectrum of the reaction mixture showed no band near 325 mμ characteristic of a 7-hydroxycoumarin. It is possible that elimination of the amino group had occurred since no starting material (71) was recovered. In support of this postulate, previous workers found that electron-withdrawing substituents on the α-position of acetoacetic esters undergo elimination rather than condensation (54, 55).

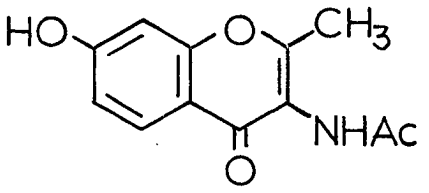
A neutral protecting group on the amine appears to be necessary for condensation to occur readily. Since the N-acetyl derivative (73) can be readily synthesized, the scheme (73 → 76) was proposed. Condensation of 73 with resorcinol and polyphosphoric acid or zinc chloride in ethanol gave only starting materials. Since the sulfuric acid method affords low yields with substituted acetoacetic esters, an alcoholic hydrogen chloride solution was used to give the desired product, 3-acetamido-7-hydroxy-4-methylcoumarin (74). Although this condensation can proceed to give either a 7-hydroxycoumarin (74) or a 7-hydroxychromone (74a) only the former is observed as demonstrated by the ir and uv spectra. The ir spectrum of the coumarin ring system shows carbonyl stretching (1720-1740 cm^{-1}) while the chromone ring system shows carbonyl absorption (1650-1680 cm^{-1}). The uv spectrum

Figure 8. Synthesis of 7-hydroxy-3-isothiocyanato-4-methylcoumarin (76).


CH3CCH2CO2Et + HNO2
69
CH3CCCO2Et
N
OH
70
Zn
HOAc
Ac2O
SnCl2
HCl
CH3CCHCO2Et
HNAc
73
CH3CCHCO2Et
NH3+Cl−
71
resorcinol
MeOH,HCl
resorcinol
H2SO4
HO
O
O
NHAc
CH3
74
HO
O
O
+NH3
CH3 Cl−
72
H3O+
HO
O
O
NH2
CH3
75
CSCl2
HO
O
O
NCS
CH3
76



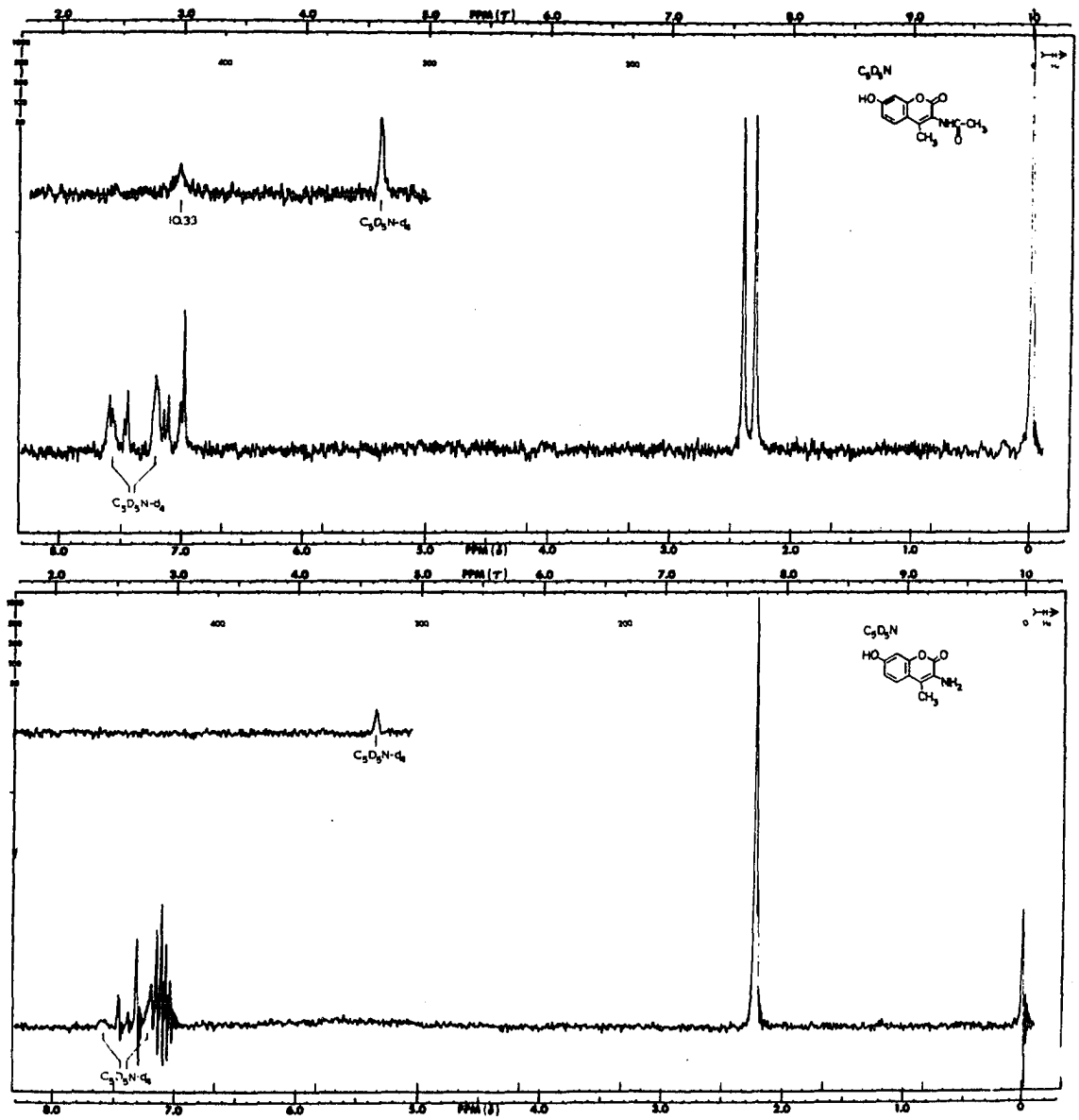
74



74a

of the 7-hydroxycoumarins is characterized by an absorption at λ_{max} 325 mμ while the 7-hydroxychromones show absorption at λ_{max} 250 mμ (85). The ir spectrum of 74 showed carbonyl absorption (1720 and 1655 cm^{-1}) as well as O-H and N-H stretching frequencies (3325 and 3135 cm^{-1}). The uv spectrum showed λ_{max} 324 mμ (log. 4.27) in methanol and λ_{max} 375 mμ (log. 4.37) at pH 10. The nmr spectrum of 74 (Figure 9) indicated a C_4 methyl group (2.29 ppm), an N-acetate (2.4 ppm), 3-aromatic protons and no olefinic proton. The complex aromatic region is an ABX pattern in which $J_{BX} \cong 0$. The C_5 proton (7.5 ppm, J_{56}=9 Hz) is coupled to the C_6 proton (7.05 ppm, J_{56}=9 Hz) and the C_6 proton is further split by the C_8 proton (6.98 ppm, J_{68} = 1.5 Hz). The aromatic region is further complicated by solvent (D_5-pyridine) which has isotope impurities at 433, 454 and 573 cps. The phenolic proton was observed at 10.33 ppm while the amide proton was found by integration in the region of 5.0-4.0 ppm.

Figure 9. Nuclear magnetic resonance spectra.
a: 3-Acetamido-7-hydroxy-4-methylcoumarin (74).
b: 3-Amino-7-hydroxy-4-methylcoumarin (75).


PPM (τ)
PPM (δ)
10.33
C_5D_5N-d_6
C_5D_5N
HO
O
NH-C-CH$_3$
CH$_3$
NH$_2$

Acid hydrolysis of 74 afforded 3-amino-7-hydroxy-4-methylcoumarin (75). The ir spectrum of 75 showed carbonyl absorption (1668 cm^{-1}) and O-H and N-H stretching frequencies (3450 and 3200 cm^{-1}). The nmr spectrum (Figure 9) demonstrated the loss of N-acetate (2.4 ppm). It also showed a low broad 3-proton absorption at 6.5-4.5 ppm (by integration). This absorption was assigned to the phenolic and amine protons since the absorption disappeared on adding D_2O. The aromatic pattern has become more complex with the chemical shift of the C_5 proton located at higher field (7.38 ppm) than in 74. This was expected since the electron density is now higher at this position due to electron donation from the 3-amino group. A similar shift in position of the C_5 proton is noted for 8-amino-7-hydroxy-4-methylcoumarin for the same reason. The fact that a shift to higher field is noted for C_5 proton adds further proof to the coumarin ring system. If a chromone ring system had been formed, no shift in the C_5 proton would be expected (no direct resonance) on hydrolysis of the amide.

Condensation of 75 with thiophosgene afforded 7-hydroxy-3-isothiocyanato-4-methylcoumarin (76). The ir spectrum of 76 showed a strong isothiocyanate band (2000 cm^{-1}), carbonyl (1695 cm^{-1}) and O-H stretching absorptions (3240 cm^{-1}). The fluorescence spectrum showed a single band (465 mμ) when excited at 385 mμ at pH 10. The intensity of fluorescence is diminished considerably from that observed for

7-hydroxy-4-methylcoumarin but is considerably higher than 8-isothiocyanate derivatives 59 and 62.

Extension of conjugation and the ability of a phenyl ring to act as an electron sink prompted the investigation of the 3-phenyl derivative. Electron-withdrawing substituents in the 3-position are known to increase the fluorescence and it was postulated that extension of conjugation would shift the fluorescence maximum to the red which is more desirable. A synthetic scheme for the preparation of 7-hydroxy-3-(p-isothiocyanatophenyl)-4-methylcoumarin (79) was devised (Figure 10). The p-nitrophenylsubstituent was introduced by allowing diazotized p-nitroaniline to react with 7-hydroxy-4-methylcoumarin by the method of Sawhney and Seshadri (86). These authors reported 7-hydroxy-4-methyl-3-(p-nitrophenyl)coumarin (77) melted at 280-1°. The same compound, prepared in this laboratory by the same procedure was found to melt at 257-8°. This discrepancy may be due to the type of melting point apparatus employed since these authors report the melting point of 3-(p-chlorophenyl)-7-hydroxy-4-methylcoumarin as 251-2°, while Freund (87) reported 233-4° for the same compound. The ir spectrum of 77 showed hydroxyl stretching frequencies (3300 and 3200 cm^{-1}), α-pyrone absorption (1680, 1615 and 1600 cm^{-1}) and nitro absorption (1515 and 1345 cm^{-1}).

The nmr spectrum of 77 (Figure 11) confirmed the substitution at the 3-position. No olefinic proton was observed

and the fine allylic coupling to the C_4 methyl was absent. The aromatic region showed the ABX pattern of 7-hydroxy-4-methylcoumarin but superimposed on the C_5 proton was part of the A_2B_2 pattern of the para substituted phenyl group. The 1-proton broad singlet (10.67 ppm) was assigned to the phenolic proton.

Reduction of 77 with stannous chloride gave quantitative yields of 3-(p-aminophenyl)-7-hydroxy-4-methylcoumarin (78). The ir spectrum of 78 showed loss of the nitro bands and addition of an amine absorption (3300 cm^{-1}). Treatment of 78 with thiophosgene gave the corresponding isothiocyanate (79). The uv of 79 showed only a 5 mμ shift in the coumarin absorption band (323 to 328 mμ) indicating the 3-phenyl derivative contributes very little to extend the π-electron system. The fluorescence spectrum of 79 showed a single band (465 mμ) at pH 10 identical with that of 76 indicating again that the phenyl ring has no effect on extending conjugation. However, the intensity of fluorescence of 79 is 20% that of 7-hydroxy-4-methylcoumarin and 6 times that of 76. The fact that 76 and 79 show decreased fluorescence when compared with an unsubstituted compound indicates the isothiocyanato group has an electron-donating effect. This suggests that an unconjugated phenylisothiocyanate group would show fluorescence intensity and color very similar to 7-hydroxy-4-methylcoumarin.

Separation of the phenyl ring from the coumarin nucleus by a methylene group required the preparation of the

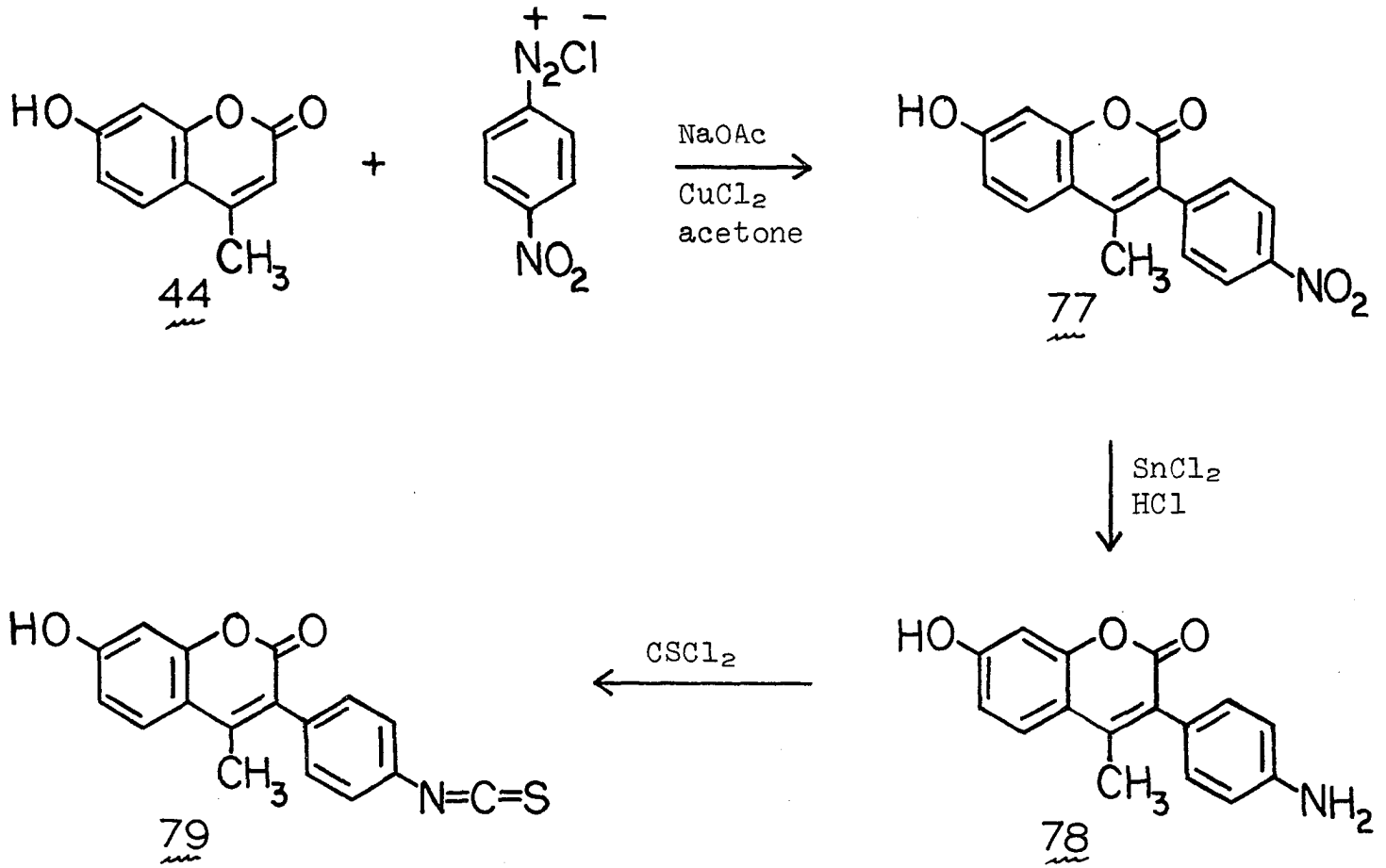
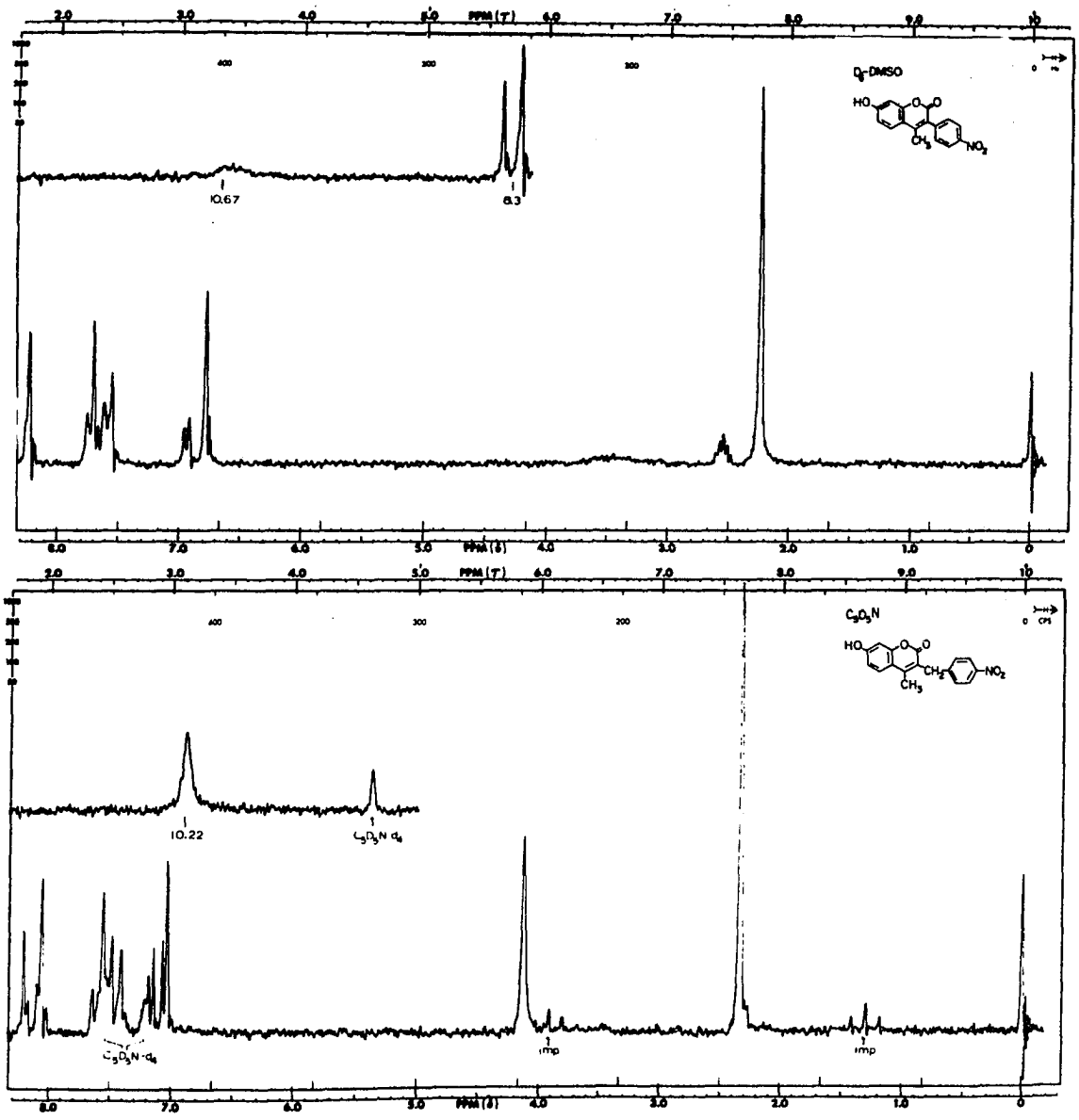
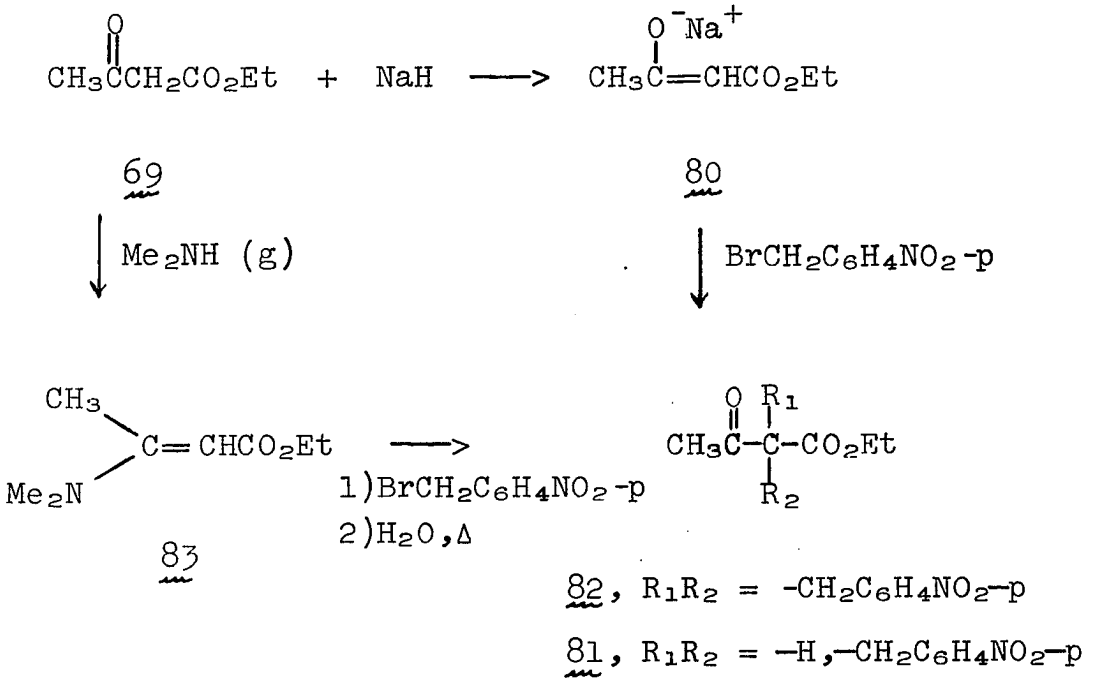


Figure 10. Synthesis of 7-hydroxy-3-(p-isothiocyanatophenyl)-4-methyl-coumarin (79).

Figure 11. Nuclear magnetic resonance spectra.
a: 7-Hydroxy-4-methyl-3-(p-nitrophenyl)coumarin (77).
b: 7-Hydroxy-4-methyl-3-(p-nitrobenzyl)coumarin (84).


PPM (τ)
PPM (δ)
D_6-DMSO
HO
CH_3
NO_2
1
10.67
1
8.3
C_5D_5N
CH_2
1
10.22
1
C_5D_5N d_5
C_5D_5N-d_5
1
imp
1
imp

corresponding α-substituted ethyl acetoacetate. The simplest system, ethyl α-(p-nitrobenzyl)acetoacetate (81) was prepared by the method of Clark and Johnson (88) from the sodium salt of ethyl acetoacetate (80) and p-nitrobenzylbromide. Unfortunately, a mixture of mono (81) and disubstituted (82) ethyl acetoacetates are formed in this sequence. A 50% yield of 81 can be obtained if a 4:1 molar ratio of ethyl acetoacetate to ethyl acetoacetate sodium salt is used with 1 mole of benzyl bromide.



In order to obtain a better yield and a simpler method of purification of 81, a new synthesis via an enamine was devised. The enamine, ethyl β-dimethylaminocrotonate (83) was prepared from 69 with dimethylamine gas by the procedure of

of Glickman and Cope (89). Condensation of 83 with p-nitrobenzylbromide gave 80% yields of the desired product (81). Condensation of 81 with resorcinol in either concentrated sulfuric acid or polyphosphoric acid gave the expected coumarin, 7-hydroxy-4-methyl-3-(p-nitrobenzyl)coumarin (84) (Figure 12). The polyphosphoric acid method is superior since the reaction is faster (0.5 hr. vs. 18 hr.) and the yields are higher (90% vs. 41%). The ir spectrum of 84 showed O-H stretching (3300 cm^{-1}), α-pyrone absorption (1675, 1600 and 1580 cm^{-1}) and nitro absorption (1518 and 1350 cm^{-1}). The nmr spectrum (Figure 11) showed the ABX pattern of a 3-substituted 7-hydroxy-4-methylcoumarin. However, superimposed on the C_5 proton is part of the A_2B_2 from the p-nitrobenzyl group. The singlets at 4.13 and 2.33 ppm are assigned to the benzylic and methyl protons, respectively. The phenolic proton is assigned to the broad singlet (10.22 ppm) which is found to disappear on adding D_2O. The uv of 84 showed λ_{max} 325 mμ in methanol and λ_{max} 370 mμ in basic solution identical to 7-hydroxy-4-methylcoumarin. This does not allow for any chromone formation which would be expected to show λ_{max} 250 mμ.

The reduction of 7-hydroxy-4-methyl-3-(p-nitrobenzyl)-coumarin with zinc dust in ethanol gave nearly quantitative yields of 3-(p-aminobenzyl)-7-hydroxy-4-methylcoumarin (85). Conversion of 85 to the isothiocyanate (86) was accomplished with thiophosgene. The ir spectrum of 86 showed strong O-H

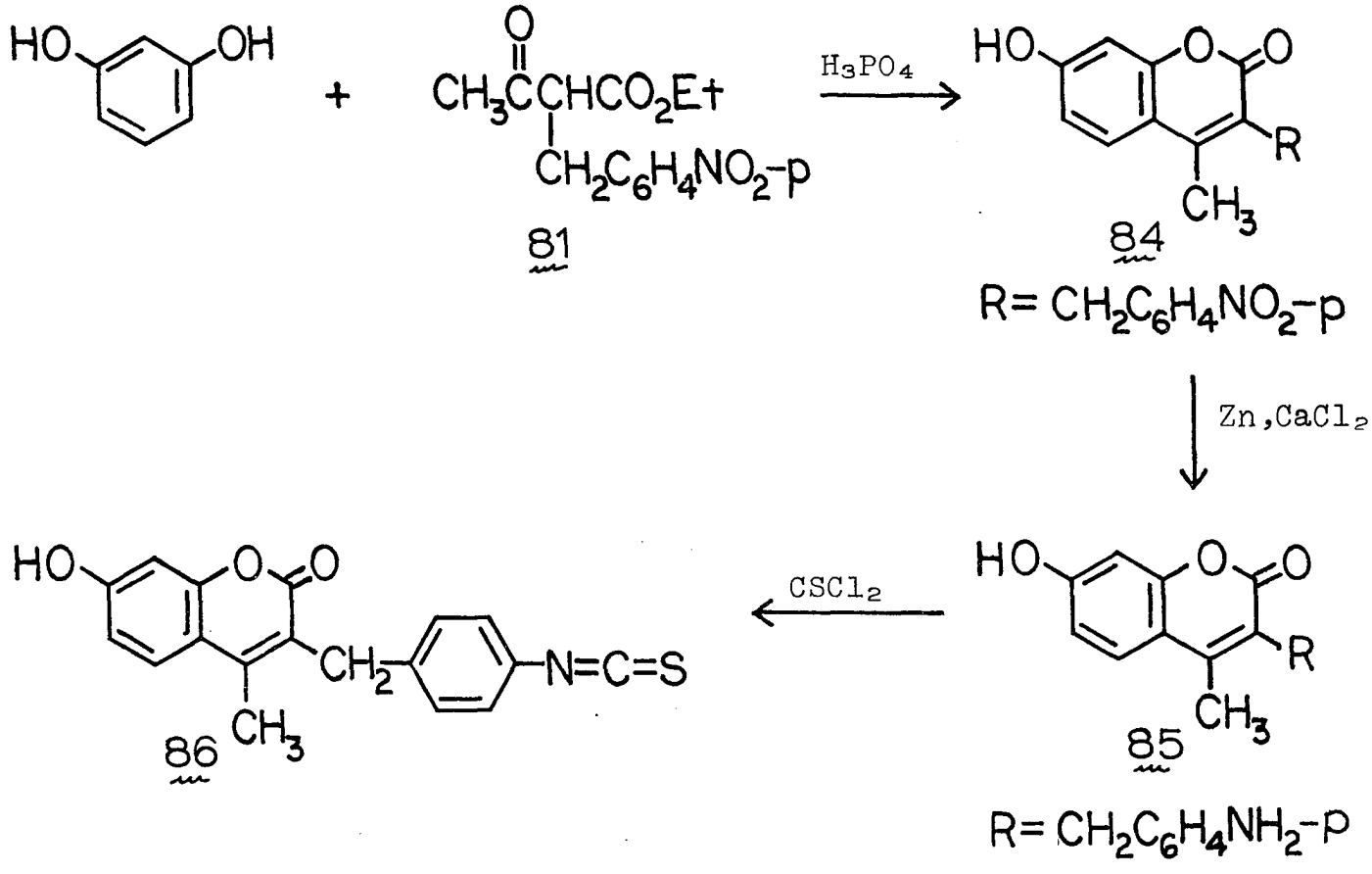


Figure 12. Synthesis of 7-hydroxy-3-(p-isothiocyanatobenzyl)-4-methylcoumarin (86).

stretching (3360 cm^{-1}), isothiocyanate absorption (2190 and 2120 cm^{-1}) and α-pyrone absorption (1682, 1620 and 1610 cm^{-1}). The fluorescence spectrum of 86 showed a single band (458 mμ) at pH 10. When compared to 7-hydroxy-4-methylcoumarin, 86 is 1.17 times as fluorescent and when compared to fluorescein isothiocyanate, 86 is 4 times as fluorescent. The intensity of fluorescence of 86 compared to the two previously prepared isothiocyanates, 76 and 79, demonstrates the effect of conjugation to the coumarin nucleus. Conjugation causes a slight bathochromic shift (465 mμ vs. 458 mμ) but conjugation also causes a decrease in fluorescence intensity.

Syntheses of 3-Substituted 7-Dimethylamino-4-methylcoumarins

It has been reported that replacement of the 7-hydroxy group with the 7-dialkylamino group displaces the fluorescence wavelength further to the red as well as increasing the intensity of fluorescence (73). These facts are important in the synthesis of fluorochromes which show color contrast to compounds already prepared (76, 79 and 86). Since the most fluorescent fluorochromes are desired, the synthesis and fluorescence data of the corresponding 3-isothiocyanato (90), 3-isothiocyanatophenyl (94) and 3-isothiocyanatobenzyl-7-diethylamino-4-methylcoumarins (97) were primary objectives.

Synthesis of the 3-isothiocyanato derivative (90) was accomplished as shown in Figure 13. Condensation of m-diethylaminophenol and ethylacetoacetate in the presence of

zinc chloride gave the 7-diethylamino derivative (87). Nitration of 7-diethylamino-4-methylcoumarin (87) with nitric acid and sulfuric acid gave the 3-nitro derivative (88) along with two dinitro derivatives, the substitution pattern of which was not determined. Mono nitration at the 3-position rather than at the 6- or 8-position is probably due to deactivation of the benzene ring upon protonation of the diethylamino group. The ir spectrum of 88 showed α-pyrone absorption (1730, 1618 and 1590 cm^{-1}) and nitro absorption (1527 and 1352 cm^{-1}). The nmr spectrum of 88 (Figure 14) showed an ABX pattern in the aromatic region, a C_4 methyl group (2.44 ppm) and no olefinic proton. This type of spectrum is characteristic of 3-substituted coumarins. In addition the nmr spectrum showed a 6-proton triplet (1.24 ppm) and a 4-proton quartet (3.46 ppm) for the methyl and methylene groups of the two N-ethyl groups.

Reduction of 88 with stannous chloride and hydrochloric acid gave a 76% yield of the 3-amino derivative (89). The ir spectrum changed accordingly, showing N-H stretching (3490 and 3400 cm^{-1}) and loss of nitro absorption (1352 cm^{-1}). The amine (89) was converted to the isothiocyanate (90) with thiophosgene in 93% yield. The loss of N-H stretching (3490 and 3400 cm^{-1}) and the addition of a broad absorption (2010 cm^{-1}) were observed in the ir spectrum of 90. The fluorescence spectrum of 7-diethylamino-3-isothiocyanato-4-methylcoumarin in methanol showed a single band (475 mμ).

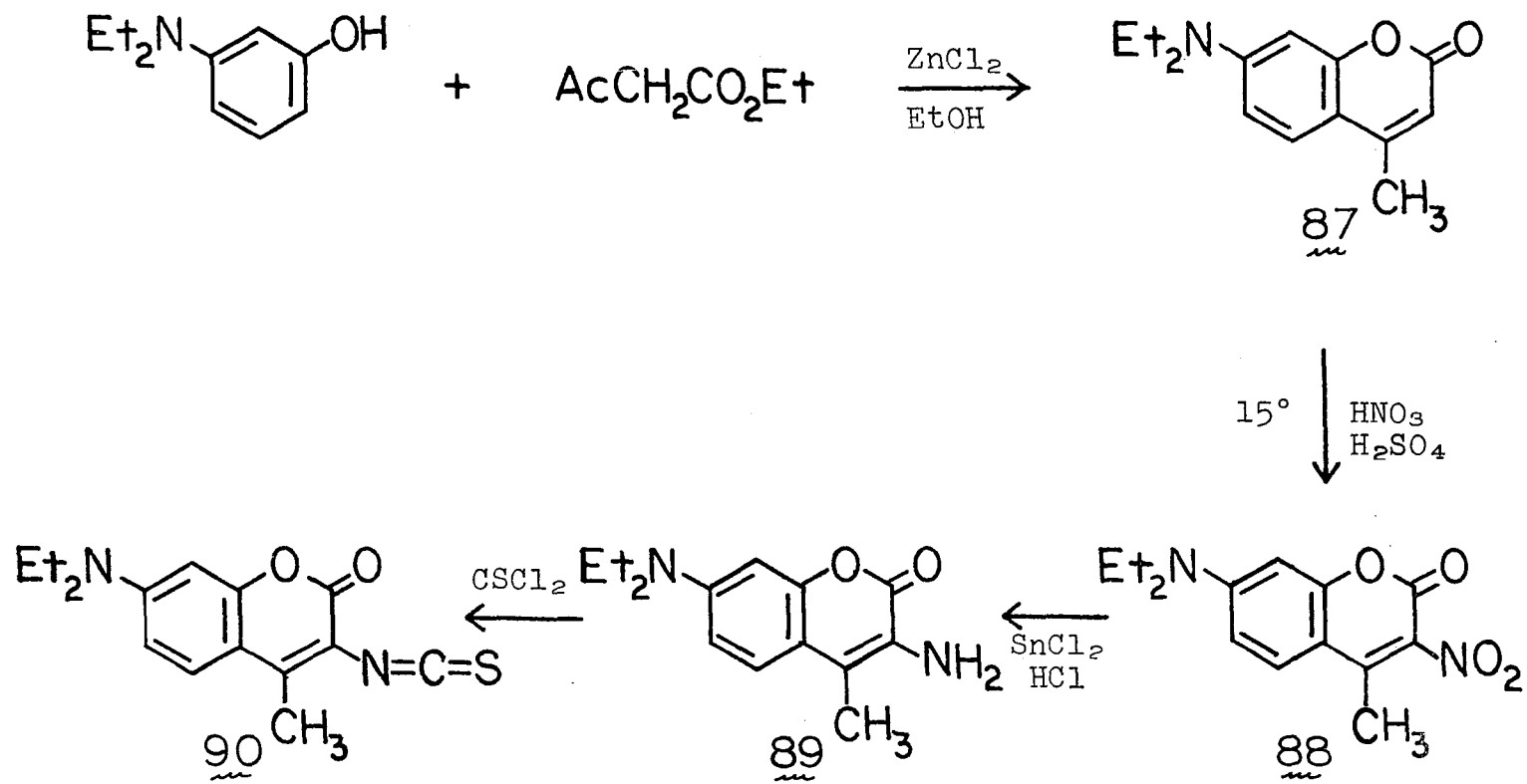
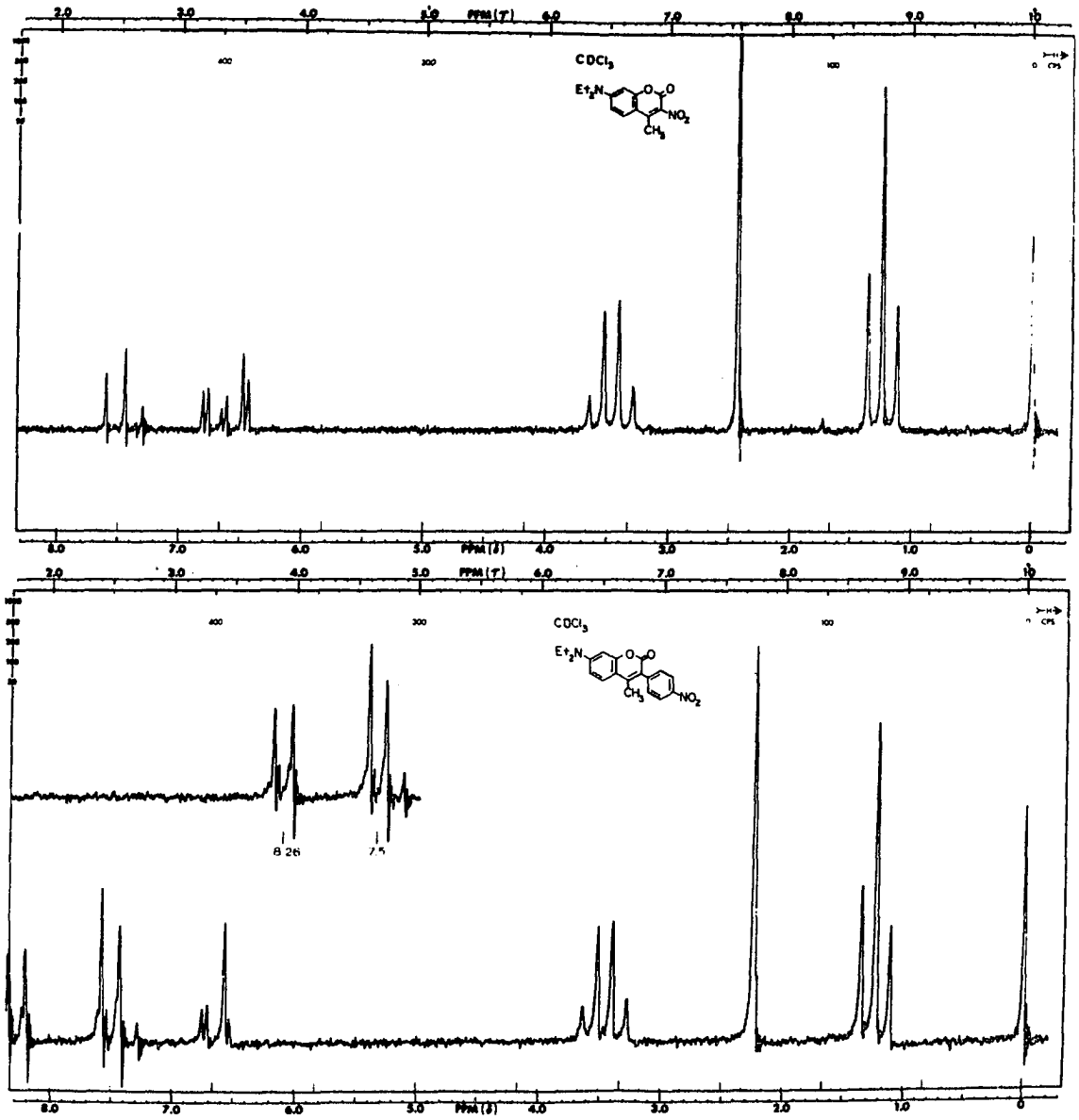


Figure 13. Synthesis of 7-diethylamino-3-isothiocyanato-4-methylcoumarin (90).

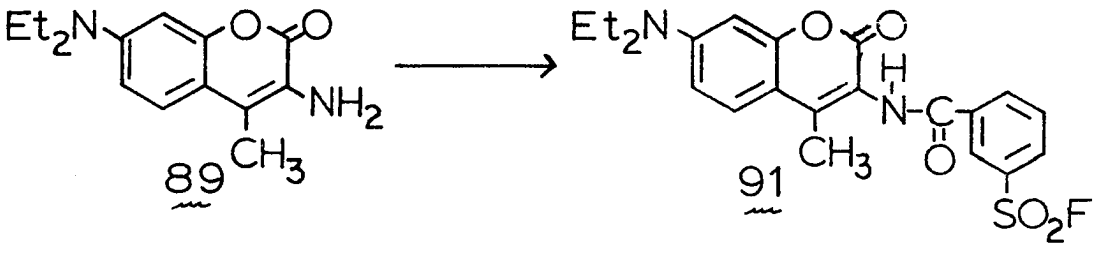
Figure 14. Nuclear magnetic resonance spectra.
a: 7-Diethylamino-4-methyl-3-nitrocoumarin (88).
b: 7-Diethylamino-4-methyl-3-(p-nitrophenyl)-coumarin (92).


CDCl3
Et2N
NO2
CH3
8.26
7.5
PPM (δ)
PPM (τ)

This corresponds to 10 mμ bathochromic shift when compared to 76. The intensity of fluorescence was found to be 22% of 7-hydroxy-4-methylcoumarin, but approximately 6 times the fluorescence intensity of the 7-hydroxy derivative (76). This intensity corresponds almost exactly to that observed for tetramethylrhodamine isothiocyanate.

In order to determine whether a benzoyl group causes quenching when not in the proximity of a hydroxy group, the synthesis of 7-diethylamino-3-[m-(fluorosulfonyl)benzamido]-4-methylcoumarin (91) was proposed. 3-Amino-7-diethylamino-4-methylcoumarin was treated with m-(fluorosulfonyl)benzoyl chloride in DMF to give 91. The ir spectrum of 91 showed N-H stretching (3390 and 3290 cm^{-1}), broad carbonyl absorption (1690 cm^{-1}) and α-pyrone absorption (1628 and 1605 cm^{-1}). The fluorescence spectrum of 91 showed three bands (378, 400 and 422 mμ) in methanol, of which the 400 mμ band was the most intense. The intensity of fluorescence was found to be diminished considerably from the corresponding isothiocyanate (90) and found to be less than 1% the intensity of 7-hydroxy-4-methylcoumarin. The fact that the intensity of fluorescence is not increased when the 7-hydroxyl is replaced with a 7-diethylamino group suggests that regardless of the group attached to the coumarin nucleus, quenching will still be a major problem with the benzoyl group.

Since it was found that 79 was 6 times as fluorescent as 76 in the 7-hydroxy series, we expected the corresponding



7-diethylamino-3-(p-isothiocyanatophenyl)-4-methylcoumarin (94) to be more fluorescent than 90. The synthesis of 94 was carried out in a manner very similar to that of 79 (Figure 15). 7-Diethylamino-4-methyl-3-(p-nitrophenyl)coumarin (92) was prepared by coupling diazotized p-nitroaniline with 87. Although 92 was previously reported, the authors (90) did not report their yield or melting point for the compound. The ir spectrum of 92 showed α-pyrone absorption (1700, 1625 and 1595 cm^{-1}) and nitro absorption (1525 and 1352 cm^{-1}). The nmr spectrum of 92 (Figure 14) showed a complex pattern in the aromatic region. Superimposed on the original ABX pattern of 7-hydroxy-4-methylcoumarin is an A_2B_2 pattern assigned to the 4-protons on the p-nitrophenyl group. Substitution at the 3-position is demonstrated by the loss of the olefinic proton and loss of the fine allylic coupling to the C-4 methyl group.

Reduction of 92 with zinc and calcium chloride in refluxing ethanol gave an 80% yield of 3-(p-aminophenyl)-7-diethylamino-4-methylcoumarin (93). The amine (93) was converted to the isothiocyanate (94) in 96% yield with

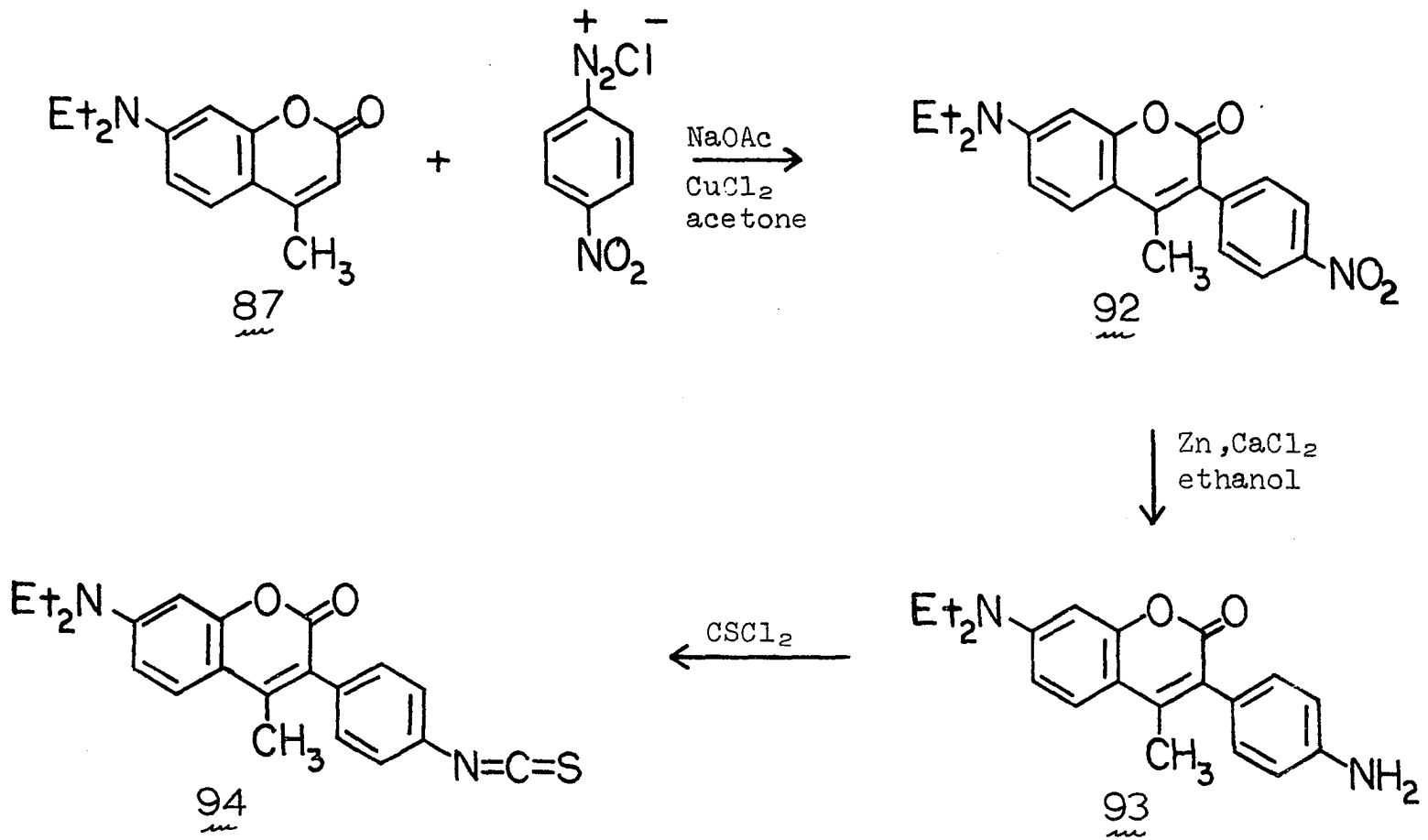
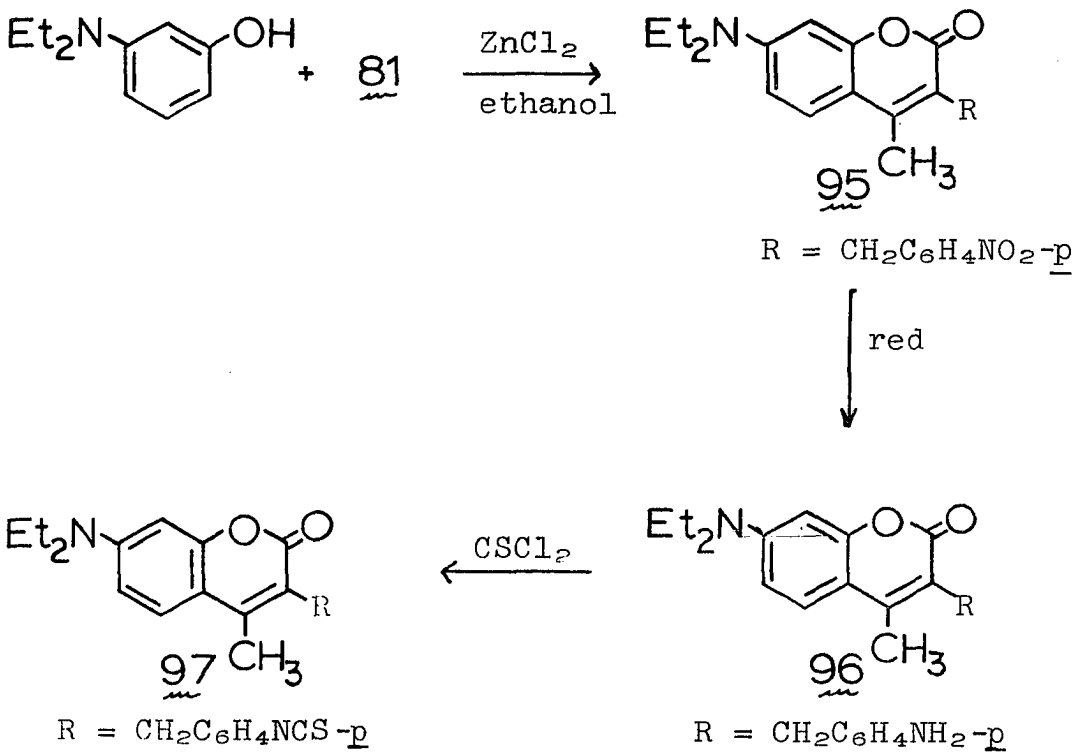


Figure 15. Synthesis of 7-diethylamino-3-(p-isothiocyanatophenyl)-4-methyl-coumarin (94).

thiophosgene. The ir spectrum of 94 demonstrated an isothiocyanate (2190 and 2120 cm^{-1}) and α-pyrone absorption (1695, 1610 and 1590 cm^{-1}). The fluorescence spectrum showed a single band (470 mμ) in methanol. The intensity of fluorescence was found to be 1.7 times that of 7-hydroxy-4-methylcoumarin and 6.1 times that of fluorescein isothiocyanate.

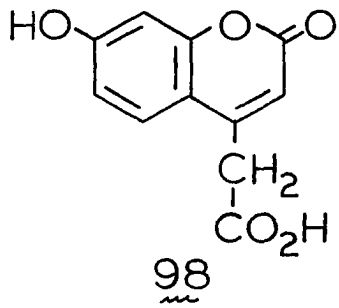
This series appeared very promising and the benzyl derivative (97) was expected to be even more fluorescent than 94. However, attempts to prepare 7-diethylamino-3-(p-isothiocyanatobenzyl)-4-methylcoumarin (97) by the sequence (95 → 97) were unsuccessful.



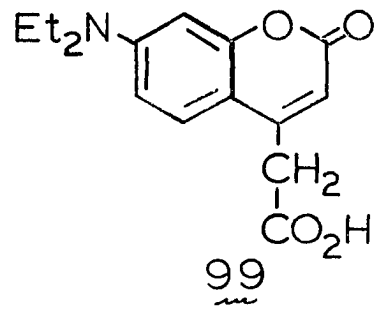
Condensation of 81 with m-diethylaminophenol and zinc chloride, polyphosphoric acid or alcoholic hydrogen chloride was unsuccessful. In each case an oil was isolated which failed to indicate a coumarin band in the uv at approximately 380 to 410 mμ. Apparently large bulky groups in the α-position of the acetoacetic ester inhibit condensation with m-diethylaminophenol.

Of the fluorochromes prepared, the 7-hydroxy derivatives 76, 79 and 86 are soluble in basic solution (pH 10) but the 7-diethylamino derivatives 90 and 94 are only soluble in organic solvents. These circumstances cause some problems since the reaction of the fluorochrome with protein is best carried out in aqueous media. The reaction may be accomplished in acetone-water or DMSO-water mixtures, but denaturation of the protein with excess acetone and removal of DMSO presents some problems. It would be advantageous to have a strongly fluorescent fluorochrome which would be soluble in aqueous or slightly basic solutions.

Mattoo (72) reported that 7-hydroxycoumarin-4-acetic acid (98) was 1.5 times as fluorescent as 7-hydroxy-4-methylcoumarin. By analogy, it was expected that 7-diethylaminocoumarin-4-acetic acid (99) would also be strongly fluorescent and soluble in dilute basic solution. Substitution of an isothiocyanatophenyl group in the 3-position of 99 would be expected to make an ideal fluorochrome.

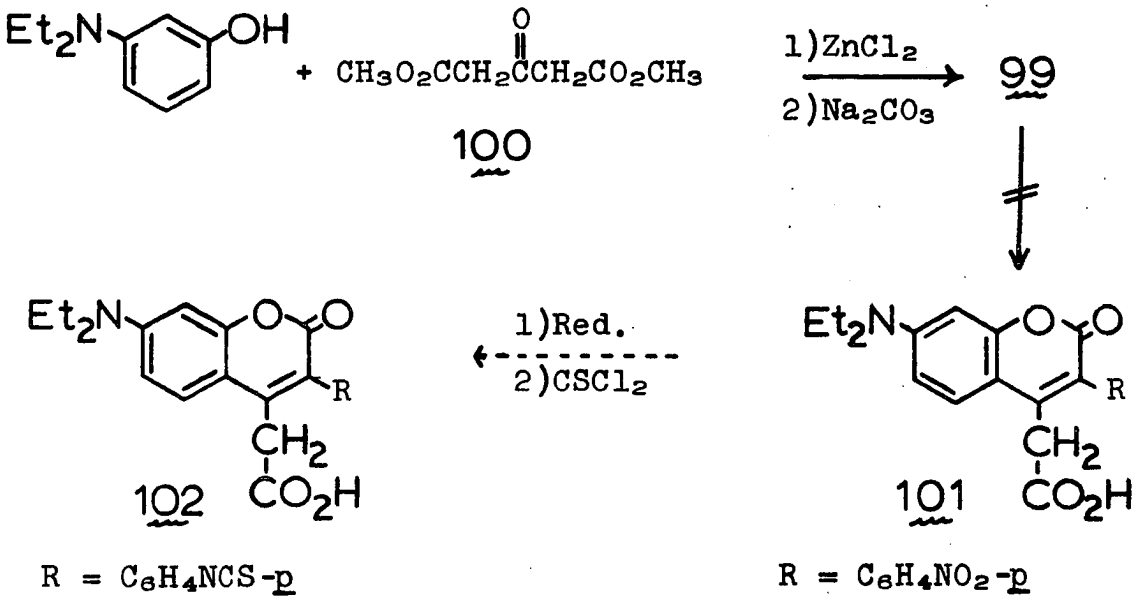


98



99

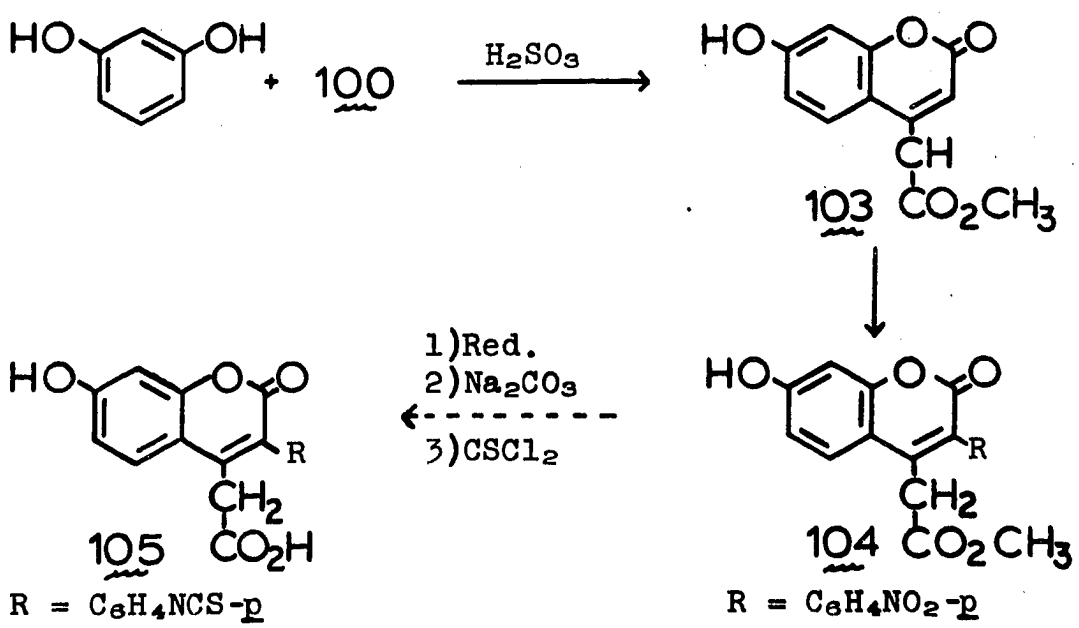
The synthesis of 7-diethylamino-3-(p-isothiocyanatophenyl)coumarin-4-acetic acid (102) was expected to be analogous to the preparation of 79 and 94. 7-Diethylaminocoumarin-4-acetic acid methyl ester was prepared by condensing m-aminophenol with dimethylacetonedicarboxylate (100) in ethanol with zinc chloride as a catalyst. The resulting oil was very difficult to purify. The residue was treated with alcoholic sodium carbonate and the acid (99) was isolated. Coupling with diazotized p-nitroaniline was not successful. Attempts to prepare the 3-(p-nitrophenyl) derivative (101) resulted only in the formation of tar. The sequence (100 → 102) may still be useful if 7-diethylaminocoumarin-4-acetic acid methyl ester is used instead of the acid (99). This postulate has added support, since it was found that the 7-hydroxycoumarin-4-acetic acid methyl ester (103) does undergo coupling with diazotized p-nitroaniline. Although the complete sequence (103 → 105) was not completed, the utility of 105 as a good fluorochrome is enhanced by the



R = C_6H_4NCS-p

R = $C_6H_4NO_2$-p

fact that 98 shows fluorescence intensity 1.5 times that of 7-hydroxy-4-methylcoumarin (72).



R = C_6H_4NCS-p

R = $C_6H_4NO_2$-p

Fluorescence of Substituted 7-Hydroxy and 7-Diethylamino-4-methylcoumarins

The fluorescence of 7-hydroxy and 7-diethylamino-4-methylcoumarins is dependent on the functional group attached to the ring system as well as the position of attachment. No fluorescence was observed for the nitro derivatives regardless of position of attachment. This fact is in accord with the results of previous workers, who found that nitro groups commonly produced complete quenching of fluorescence. Direct photodissociation or predissociation was suggested as a major factor responsible for the quenching action of the nitro group (91).

The amino derivatives (Table 3) show fluorescence in the blue and blue-green region of the visible spectrum. However, in all cases, the intensity of fluorescence is lower than the corresponding unsubstituted compounds, 7-hydroxy-4-methylcoumarin and 7-diethylamino-4-methylcoumarin. In general, it is noted that substituents which act as conjugative electron donors often increase the fluorescence yield of an aromatic system. It has been postulated that the principal effect of a conjugatively electron-donating substituent is simply to increase radiative transition probabilities, between ground state singlet (S_o) and excited state singlet (S_1^*), so that emission competes more effectively with radiationless deactivation. However, in the case

of amines and other strongly electron-donating substituents lower emission yields are frequently found. It is likely that at least part of that substituent effect results from a change in the probability of $S_o \Longleftrightarrow S_1^*$ radiative transitions (75).

Conversion of the amine to the amide shows some conflicting results. It would be expected that the amide would show much stronger fluorescence than the corresponding amine since its electron donation ability is considerably diminished. This postulate is substantiated by the 8-acetamido (54) and the 3-acetamido (74) derivatives which show a ten-fold increase in fluorescence intensity over the corresponding amines. However, the 8-benzamido (66) and the 3-benzamido (91) derivatives show no increase in fluorescence intensity over the corresponding amines. This may be explained on the basis of some excited singlet state. It has been reported (75) that most aromatic carbonyl compounds possess lowest excited singlet states of (n, π^*) character, therefore, intersystem crossing to the triplet manifold is usually very efficient. For this reason a large number of aromatic carbonyl compounds exhibit fairly intense phosphorescence but no fluorescence. A similar phenomenon may be occurring with the benzamides. Apparently, the fluorosulfonyl group exerts little effect on the quenching ability of the benzamides since both the unsubstituted benzamide (67) and 3-benzoyl-7-hydroxycoumarin (68) show similar low intensity fluorescence.

The fluorescence of the isothiocyanates (Table 4) showed intensities of greater magnitude than the corresponding

amines (Table 3) but of lower intensities than the acetamides. This suggests that the isothiocyanate group has some conjugative electron-donating ability but of decreased intensity compared to the amino group. No sigma values were found for the isothiocyanate group to support or refute this postulate.

Due to the synthetic difficulties encountered in preparing isothiocyanates substituted on the aromatic ring and the apparent lower fluorescence intensities of these derivatives, it appears that coumarins substituted in the pyrone ring would make the best fluorochromes. Although the fluorescence intensity increases in the series 7-hydroxy-3-isothiocyanato-, 7-hydroxy-3-(p-isothiocyanatophenyl)- and 7-hydroxy-3-(p-isothiocyanatobenzyl)-4-methylcoumarin by a factor of 33, there is little change in the wavelength of the fluorescence maximum. This fact is in accord with the results of previous workers (74) who recently found that 7-hydroxy-3-phenylcoumarin, 7-hydroxy-3-acetylcoumarin and 7-hydroxy-3-cyanocoumarin did not show a shift in the wavelength of the fluorescence maximum. Apparently substituents in the 3-position of 7-hydroxycoumarins serve only to stabilize or destabilize the electron shift into the carbonyl of the pyrone ring.

The 7-diethylamino-4-methylcoumarin derivatives show increased fluorescence intensity and a slight shift in the wavelength of the fluorescence maximum to the red compared to the corresponding 7-hydroxy-4-methylcoumarin derivatives.

Table 3. Fluorescence of amino 4-methylcoumarins

4-Methylcoumarin	Absorption λ_{max}, (mμ); log ε		Excitation λ_{max} (mμ)	Fluorescence λ_{max} (mμ)	Rel. Fluorescence intensity[a,b]	pH
7-Hydroxy (44)	322	4.17	320	385	221	7
	373	4.33	370	445	1085	10
8-Amino-7-hydroxy (47)	319	4.17	---	---	---	4
	325	4.11	---	---	---	7
	284	4.08	323	458	nil	10
8-Amino-7-methoxy-methyl (58)	312	4.02	360	398	0.73	7
	309	4.07	323	460	1.17	4
3-Amino-7-hydroxy (75)	326	4.23	340	455	31.8	4
	334	4.26	305	360	123.0	7
	365	4.20	377	480	6.5	10
3-(p-Aminophenyl)-7-hydroxy (78)	327	4.31	330	450	0.10	4
	331	4.27	320	390	4.4	7
	370	4.44	375	450	0.87	10
3-(p-Aminobenzyl)-7-hydroxy (85)	323	4.24	---	---	---	4
	323	4.24	360	380	4.9	7
	365	4.36	377	458	25.4	10
3-Amino-7-diethylamino (89)	395	3.38	385	495	17.7	4
	378	4.24	388	477	272.0	7
3-(p-Aminophenyl)-7-diethylamino (93)	380	3.72	402	480	85.0	4
	380	4.48	395	475	10.0	7

[a]Fluorescence intensities observed at concentration of 1×10^{-2} mole/l.

[b]Relative intensities were corrected for variations in source intensity, grating transmission, or photo multiplier sensitivity (92).

Table 4. Fluorescence of substituted 4-methylcoumarin

4-Methylcoumarin	Absorption λ_{max},(mμ);log ε		Excitation λ_{max}(mμ)	Fluorescence λ_{max} (mμ)	Rel. Fluorescence intensity[a,b]	pH
7-Hydroxy (44)	322	4.17	320	385	18.0	7
	373	4.33	370	445	87.0	10
8-Acetamido-7-hydroxy (54)	321	4.16	335	437	0.65	7
	374	4.31	360	463	17.7	10
8-Isothiocyanato-7-methoxy (59)	306	4.29	350	410	nil	7
8-[m-(Fluorosulfonyl)-benzamido]-7-hydroxy (66)	320	4.18	348	395	0.04	7
	367	4.29	375	460	0.18	10
8-Benzamido-7-hydroxy (67)	320	4.15	330	390	0.05	7
	369	4.30	330	475	0.10	10
3-Acetamido-7-hydroxy (74)	324	4.27	335	400	38.2	7
	375	4.37	380	460	92.5	10
7-Hydroxy-3-isothiocyanato (76)	347	4.45	415	470	0.11	7
	395	4.52	385	465	3.00	10
7-Hydroxy-3-(p-isothiocyanatophenyl) (79)	328	4.75	345	425	0.43	7
	370	4.78	370	465	18.7	10
7-Hydroxy-3-(p-isothiocyanatobenzyl) (86)	323	4.61	320	400	18.2	7
	365	4.66	375	458	102.0	10

[a]Fluorescence intensity observed at concentration of 4.0×10^{-6} mole/l.

[b]Relative intensities were corrected by variations in source intensity; grading transmission or photomultiplier sensitivity (92).

Table 4 continued

4-Methylcoumarin	Absorption λ_{max},(mμ);log ε		Excitation λ_{max} (mμ)	Fluorescence λ_{max} (mμ)	Rel. Fluorescence intensity[a,b]	pH
7-Diethylamino (87)	370	4.36	388	450	41.8	7
	370	3.42	388	468	3.8	4
7-Diethylamino-3-isothiocyanato (90)	412	4.59	415	475	19.2	7
	405	4.38	425	490	8.0	4
7-Diethylamino-3-[m-(fluorosulfonyl)benzamido] (91)	249	4.60	250	380,400,422	0.35	7
	380	4.47	360	380,400,422	0.09	7
	310	4.08	320	440	0.09	4
	383	3.75	405	475	0.10	4
7-Diethylamino-3-(p-isothiocyanatophenyl) (94)	385	4.86	397	470	150	7
Fluorescein isothiocyanate (8b)			360	518	8.0	7
			360	520	25	10
			495	520	55	7
			495	518	330	10
Tetramethylrhodamine isothiocyanate (12)			360	568	19.3	7
			360	580	9.7	10
			545	565	198	7
			555	575	134	9

This may be due to the increased electron donating ability of the diethylamino group compared to the hydroxy group resulting in increased electron density in the pyrone ring. Both the 3-isothiocyanato (90) and the 3-(p-isothiocyanatophenyl) (94) derivatives show increased fluorescence intensity compared to the 7-hydroxy derivatives 76 and 79. The 7-diethylamino-4-methylcoumarin derivatives show maximum fluorescence in neutral solutions while the 7-hydroxy-4-methylcoumarin derivatives show maximum fluorescence in basic solution (pH 9-10). This may be important in determining which fluorochrome to use if a specific protein is sensitive to pH change.

Comparison of the coumarin isothiocyanates with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate is illustrated in Table 4. It must be noted that both fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate have their fluorescence maximum excited by two or more absorption bands. However, only the 360 mμ excitation band may be used since the longer wavelength bands (495 and 555 mμ) are too close to the fluorescence band (518 and 575 mμ) to allow for complete filtering. This results in a loss of potential fluorescence intensity of 94% for fluorescein isothiocyanate and 90% for tetramethylrhodamine isothiocyanate.

Table 4 indicates a number of coumarin isothiocyanates have fluorescence intensity greater than these known fluorochromes. Both 7-hydroxy-3-(p-isothiocyanatophenyl)-4-methyl-

coumarin (79) and 7-diethylamino-3-isothiocyanato-4-methylcoumarin (90) are equal in fluorescence intensity to tetramethylrhodamine isothiocyanate and 75% as fluorescent as fluorescein isothiocyanate at optimum conditions. In addition, 7-hydroxy-3-(p-isothiocyanatobenzyl)-4-methylcoumarin (86) is 4.1 times as fluorescent as fluorescein isothiocyanate while 7-diethylamino-3-(p-isothiocyanatophenyl)-4-methylcoumarin (94) showed fluorescence intensity 6 times that of fluorescein isothiocyanate.

While fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate lose fluorescence intensity upon conjugation to the protein, the coumarin isothiocyanates (76, 79, 90 and 94) are expected to increase in fluorescence intensity. The increase is expected since electron-donating groups at the 3-position cause a decrease in fluorescence intensity, while neutral or electron-withdrawing groups at the 3-position increase the fluorescence intensity. The isothiocyanate group appears to be an electron donor, however, conjugation to the protein will result in the formation of a neutral substituent (thiourea) and therefore, an increase in fluorescence intensity. In support of this postulate, it was found that the N-acetyl derivative 74 is 30 times more fluorescent than the corresponding isothiocyanate 76.

Although the blue or blue-green fluorescence of the coumarin fluorochromes is a disadvantage in tissue cultures with blue autofluorescence, it may be used to an advantage in

other cases. In particular, the coumarin fluorochromes may be used in double tracing experiments with either the orange red fluorochromes, tetramethylrhodamine isothiocyanate, or the green fluorochrome, fluorescein isothiocyanate. They also show great potential in bacterial cultures which have little background fluorescence, in tissue cultures with yellow or red autofluorescence and in tissue cultures to which a counterstain has been applied.

The strong fluorescence of the coumarins in the pH range 7 to 10, the large difference between absorption maximum and fluorescence maximum (60-80 mμ), the fluorescence excited by light of wavelength 365 mμ, the principal line of the mercury spectrum, and the relative ease of preparation suggest that these compounds should be useful fluorochromes.

SUMMARY

7-Hydroxy and 7-diethylaminocoumarins were selected as likely fluorochromes for tagging protein in antibody-antigen reactions due to their intense fluorescence in neutral and aqueous solutions. In the course of this study, a number of new derivatives of 7-hydroxy- and 7-diethyl-4-methyl-coumarin were synthesized. The methods of synthesis for 3- and 8-substituted isothiocyanato, p-isothiocyanatophenyl and p-isothiocyanatobenzyl derivatives are discussed.

Fluorescence intensities were measured for a number of amino, N-acetyl, N-benzamido and isothiocyanato coumarins. Comparison of these spectra supports the view that electron donating substituents on the 7-hydroxy and 7-diethylamino-coumarin nucleus at the 3-, 6- or 8-positions reduce the intensity of fluorescence. In contrast, neutral or electron-withdrawing substituents at the 3-position enhance the fluorescence intensity.

Evidence is presented for a number of coumarin fluoro-chromes which show equal or greater fluorescence intensity than fluorescein isothiocyanate when condensed with protein.

EXPERIMENTAL

The nuclear magnetic resonance spectra were obtained on a Varian A-60 spectrometer operating at 60 Mc.p.s. The infrared spectra were obtained with a Beckman Model IR-12 and the Perkin-Elmer Model 21 spectrophotometers. All mass spectra were determined with an Atlas CH-4 mass spectrometer using the TO-4 ion source (70 e.v.). Melting points were observed on a Kofler microscope hot-stage and are corrected. Ultraviolet spectra were obtained either on a Beckman DK-2 ultraviolet-visible or Cary Model 14 spectrophotometer. Emission spectra were obtained on a Aminco-Bowman spectrophotofluorometer. The instrument was equipped with an Osram XB165 xenon arc, a 1P28 photomultipler tube and measurements were made using slit arrangement 3. The elemental analyses were carried out by Ilse Beetz Microanalytical Laboratory, Kronach, West Germany. Thin-layer chromatography was performed on Silica Gel PF 254 + 366 (Merck) using ultraviolet light of the appropriate wavelengths. The proof of identity of two compounds was carried out by comparison of melting points and mixed melting points as well as infrared spectra and chromatographic characteristics.

The fluorescence measurements were made at 4.0×10^{-6} M unless otherwise mentioned. The solvents used were absolute methanol (Baker), potassium biphthalate buffer (pH 4.0) (Fisher), potassium carbonate-potassium borate-potassium hydroxide buffer (pH 10.0) (Fisher).

7-Hydroxy-4-methylcoumarin and 8-Substituted Derivatives

7-Hydroxy-4-methylcoumarin (44)

7-Hydroxy-4-methylcoumarin was prepared by the method outlined in Organic Synthesis (93). Condensation of 15.6 g resorcinol and 18.5 g ethyl acetoacetate gave 17.5 g (70%) of colorless needles when recrystallized from ethanol: mp 184-5° [lit. (93) mp 185]; ir (KBr) 3500 cm^{-1} (O-H), 1670, 1605 cm^{-1} (α-pyrone); λ_{max} (95%) EtOH) 322 mμ (log ϵ = 4.17); λ_{max} (1N NaOH) 373 mμ (log ϵ = 4.33); λ^{F}_{max} (MeOH) 385 mμ; λ^{F}_{max} (pH 10) 445 mμ; nmr (D_6-DMSO [Figure 5] δ 7.58 ppm (d, 1, J=9Hz, C_5-proton), 6.7 ppm (m, 2, C_6 and C_8 protons), 6.1 ppm (m, 1, olefinic proton), 2.38 ppm (d, 3, J=1.5 Hz, (CH_3).

7-Hydroxy-4-methyl-8-nitro- and 6-nitrocoumarin (45 and 46)

In a 300 ml, three-necked, round-bottomed flask, fitted with a mechanical stirrer, a thermometer and a dropping funnel was placed 10.0 g of 7-hydroxy-4-methylcoumarin and 80 ml of sulfuric acid (d 1.84). The flask immersed in an ice bath. When the temperature fell below 10°, a solution of nitric acid (4 ml, d 1.42) and sulfuric acid (12 ml, d 1.84) was added dropwise. The mixture was stirred at 5-10° until the addition was complete (1.5 hr). The reaction mixture was allowed to stand at the same temperature for 0.5 hr. Crushed ice was added and the resulting yellow precipitate was collected on a Buchner funnel and washed twice with 30 ml of water. The yellow mass was refluxed with 250 ml of 95%

ethanol for 10 min and the undissolved material was filtered. The residue was recrystallized first from acetic acid and then from benzene to give 2.3 g (18.4%) of 46 as yellow needles: mp 266-8° [lit. (76) 262°]; ir (KBr) 3450, 3250 cm^{-1} (O-H), 1725, 1635, 1580 cm^{-1} (α-pyrone), 1535, 1365 cm^{-1} (NO_2); λ_{max} (95% ethanol) 265 mμ (log ϵ = 4.40), 284 mμ (sh); 338 mμ (log ϵ = 3.98).

The alcohol filtrates were concentrated in vacuo and crystallized from acetic acid to give 5.7 g (46%) of 45 as tan prisms: mp 266-8° [lit. (94, 95) 262°]; ir (KBr) 3250 cm^{-1} (O-H), 1710, 1620 cm^{-1} (α-pyrone), 1535, 1365 (NO_2); λ_{max} (95% ethanol) 317 mμ (log ϵ = 4.12), 330 mμ (sh); nmr (D_6-DMSO) [Figure 5] δ 7.48 ppm (AB, 2, J=Hz, C_5 and C_6 protons), 6.25 ppm (m, 1, olefinic), 2.41 ppm (S, 3, CH_3).

8-Amino-7-hydroxy-4-methylcoumarin (47)

Reduction of 2.37 g 7-hydroxy-4-methyl-8-nitrocoumarin with 7.0 g of sodium hydrosulfite according to the procedure of Kaumann (76) gave 1.44 g (71%) of 47 when recrystallized from etnanol: mp 272-3° [lit. (76) 270°]; ir (KBr) 3460, 3325 cm^{-1} (O-H, N-H), 1700, 1620 cm^{-1} (α-pyrone); λ_{max} (95% ethanol) 271 mμ (log ϵ = 4.11), 325 mμ (log ϵ = 4.11); λ_{max} (1N NaOH) 284 mμ(log ϵ = 4.08); λ_{max} (1N HCl) 319 mμ (log ϵ = 4.17); nmr (D_6-DMSO) δ 6.86 ppm (4S, 2, aromatic protons), 6.1 ppm (m, 1, olefinic), 6.5-4.0 ppm (3, OH, NH_2), 2.34 ppm (d, 3, CH_3).

Condensation of thiophosgene with 8-amino-7-hydroxy-4-methylcoumarin (49)

A solution of 300 mg of 8-amino-7-hydroxy-4-methylcoumarin in 50 ml of anhydrous acetone was added dropwise to a stirred solution of 0.6 ml (800 mg) thiophosgene in 15 ml of anhydrous acetone. The reaction mixture, which was protected by a nitrogen atmosphere, was stirred an additional 8 hr. The acetone-insoluble product was separated, washed with acetone and dried to give 100 mg of 8-amino-7-hydroxy-4-methylcoumarin. The acetone-soluble mixture was evaporated to dryness in vacuo and recrystallized from acetone-hexane to give 150 mg of white needles: mp 295-315° (dec); ir (KBr) 3440, 3180, 3150, 3080 cm^{-1} (N-H), 1755, 1730, 1650, 1600 cm^{-1} (α-pyrone); λ_{max} (95% ethanol) 325 mμ (log ϵ = 3.98), 290 mμ (log ϵ = 4.60), 257 mμ (log ϵ = 3.87), 251 mμ(log ϵ = 3.82), 226 mμ (log ϵ = 4.20); mass spectrum molecular ion m/e 233.

Anal. Calcd. for $C_{11}H_7NO_3S$: C, 56.66; H, 3.03; N, 6.01. Found: C, 56.50; H, 3.11; N, 5.96.

8-N,N-Diacetamido-7-acetoxy-4-methylcoumarin (52)

8-Amino-7-hydroxy-4-methylcoumarin (209 mg), acetic anhydride (5 ml) and concentrated sulfuric acid (0.5 ml) were heated on a steam bath for 5 min. The solution was allowed to cool and then poured into 25 ml of water. The resulting precipitate was filtered, washed with water and dried. Recrystallization from acetone gave 283 mg of white prisms: mp 186-7° [lit. (78) 199°]; ir ($CHCl_3$) 1745,

1736, 1726 cm^{-1} (C=O), 1610 cm^{-1} (C=C); nmr ($CDCl_3$) δ 7.5 (AB, 2, J=9Hz, aromatic protons), 6.3 ppm (m, 1, olefinic), 2.4 ppm (d, 3, CH_3), 2.3 ppm (2S, 9, $COCH_3$); mass spectrum molecular ion m/e 317.

8-Acetamido-7-hydroxy-4-methylcoumarin (54)

Acetic anhydride (1.0 ml) was added rapidly to a boiling solution of 8-amino-7-hydroxycoumarin (50 mg) in glacial acetic acid (5 ml). After 5 min., the solution was allowed to cool to room temperature. White prisms (49 mg) were deposited: mp 291-2° [lit. (78) 290°]; ir (nujol) 3450, 3315, 3200 cm^{-1} (O-H, N-H), 1705, 1615, 1585 cm^{-1} (α-pyrone), 1670 cm^{-1} (amide C=O); λ_{max} (95% ethanol) 321 mμ (log ε = 4.16), 258 mμ (log ε = 3.74); λ_{max} (1N NaOH) 374 mμ (log ε = 4.31), 276 mμ (log ε = 3.63); λ^{F}_{max} (MeOH) 437 mμ ; λ^{F}_{max} (pH 10) 463 mμ.

7-Acetoxy-4-methyl-8-nitrocoumarin (56)

A suspension of 1.0 g 7-hydroxy-4-methyl-8-nitrocoumarin, 4.0 ml of acetic anhydride and 4 drops of concentrated sulfuric acid was heated on a steam bath until solution was effected (5-10 min). The hot solution was poured into 30 ml of water. The resulting precipitate was filtered, washed with water and dried. Recrystallization of the crude product from acetic acid gave 1.07 g of 56 as white needles: mp 201-2° [lit. (95) 199°].

7-Methoxy-4-methyl-8-nitrocoumarin (57)

To a solution of 500 mg of 7-hydroxy-4-methyl-8-nitrocoumarin in 40 ml of acetone was added 0.6 ml (1.36 g) of

methyl iodide and 340 mg of potassium carbonate. The suspension was stirred for 12 hr at 25°. Water (50 ml) was added and the reaction mixture was filtered. The aqueous solution (pH 8-9) was extracted 4 times with 50 ml portions of chloroform. The chloroform extracts were dried ($MgSO_4$), filtered and concentrated to dryness in _vacuo_. Recrystallization of the residue from acetone gave 320 mg (61%) of 57: mp 229-3° [lit. (78, 95) 230°]; ir ($CHCl_3$) 1740, 1620 cm^{-1} (α-pyrone), 1540, 1360 cm^{-1} (NO_2).

8-Amino-7-methoxy-4-methylcoumarin (58)

In a 100 ml 3-necked, round-bottomed flask, fitted with a mechanical stirrer, condenser and a dropping funnel was placed 357 mg of 7-methoxy-4-methyl-8-nitrocoumarin and 15 ml of 28.4% aqueous ammonia. A solution of sodium hydrosulfite (1.11 g) in 20 ml of water was added rapidly to the stirred suspension. The reaction flask was immersed in an oil bath and the solution refluxed for 15 min with stirring. The reaction mixture was cooled and filtered. The residue was recrystallized from acetone to give 164 mg (52%) of 58: mp 165-6° [lit. (78) 161°]; ir ($CHCl_3$) 3500, 3400 cm^{-1} (N-H), 1720, 1615, 1590 cm^{-1} (α-pyrone).

8-Isothiocyanato-7-methoxy-4-methylcoumarin (59)

A solution of 75 mg 8-amino-7-methoxy-4-methylcoumarin in 5 ml of anhydrous acetone was added dropwise to a stirred solution of 0.5 ml (750 mg) thiophosgene (Aldrich) in 5 ml of anhydrous acetone at 40°. After addition was complete,

the red solution was refluxed for 5 hr and allowed to cool (with stirring) for 12 hr. The solvent and excess thiophosgene were removed under reduced pressure and the residue recrystallized from acetone to give 77 mg (85%) of 59 as white needles: mp 178-9°; ir ($CHCl_3$) 2100, 2050 cm^{-1} (N=C=S), 1740, 1610 cm^{-1} (α-pyrone); λ_{max} (95% ethanol) 306 mμ (log ϵ = 4.29), 319 mμ (sh), 284 mμ (log ϵ = 4.40); λ^{F}_{max} (MeOH) 410 mμ; nmr ($CDCl_3$) [Figure 7] δ 7.15 ppm (AB, 2, J=9Hz, aromatic protons), 6.1 ppm (m, 1, olefinic proton), 4.0 ppm (s, 3, (OCH_3), 2.4 ppm (d, 3, CH_3); mass spectrum molecular ion m/e 247.

Anal. Calcd. for $C_{12}H_9NO_3S$: C, 58.30; H, 3.67; N, 5.67. Found: C, 58.24; H, 3.82; N, 5.62.

7-Methoxymethyl-4-methyl-8-nitroumbelliferone (60)

To 7-hydroxy-4-methyl-8-nitrocoumarin (3.36 g) in 200 ml of anhydrous acetone and potassium carbonate (4.14 g) was added chloromethylmethyl ether (2.90 g) dropwise and with stirring. The suspension was allowed to stir for 2 hr and filtered. The residue was washed with hot chloroform and added to the filtrates. The filtrates were concentrated to dryness and the residue washed with ether to remove excess chloromethylmethyl ether. The residue was crystallized from acetone to give 3.73 g (93%) of 60 as colorless prisms: mp 193-4°; ir (KBr) 1740, 1625 cm^{-1} (α-pyrone), 1540, 1370 cm^{-1} (NO_2); λ_{max} (95% ethanol) 325 mμ (sh), 312 mμ (log ϵ = 4.11), 303 mμ (sh), 290 mμ (log ϵ = 4.27), 275, 228 mμ (sh); mass spectrum molecular ion

m/e 247.

Anal. Calcd. for $C_{12}H_{11}NO_6$: C, 54.34; H, 4.18; N, 5.28. Found: C, 54.22; H, 4.25; N, 5.40.

8-Amino-7-methoxymethyl-4-methylumbelliferone (61)

In a 100 ml 3-necked, round-bottomed flask, fitted with a mechanical stirrer, a condenser and a dropping funnel was placed 917 mg of 7-methoxymethyl-4-methyl-8-nitroumbelliferone in 6 ml of 10% sodium hydroxide. A solution of sodium hydrosulfite (3.0 g) in water (10 ml) was added rapidly to the stirred suspension. The mixture was refluxed for 15 min and then allowed to cool (with stirring) for 3 hrs. The suspension was collected by filtration and recrystallization from acetone to give 675 mg of 61 as light yellow prisms: mp 114-5°; ir ($CHCl_3$) 3510, 3410 cm^{-1} (NH_2), 1718, 1612, 1595 (α-pyrone); λ_{max} (MeOH) 312 mμ (log ε = 4.02), 303 mμ (sh), 266 mμ (log ε = 4.15); λ_{max} (1N HCl) 309 mμ (log ε = 4.07), 254 mμ (log ε = 3.92), 248 mμ (log ε = 3.91); nmr ($CDCl_3$) δ 2.37 ppm (d, 3, CH_3), 3.5 ppm (s, 3, OCH_3), 4.2 ppm (s, 2, NH_2) 5.26 ppm (s, 2, methylenedioxy), 6.12 ppm (m, 1, olefinic), 6.96 ppm (AB, 2, J= 9Hz, aromatic protons); mass spectrum molecular ion m/e 235.

Anal. Calcd. for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.96. Found: C, 61.33; H, 5.59; N, 6.05.

8-Isothiocyanato-7-methoxymethyl-4-methylumbelliferone (62)

To a suspension of 1.0 ml (1.54 g) thiophosgene in 30 ml of water was added powdered 8-amino-7-methoxymethyl-4-methyl-

umbelliferone (1.0 g) in the course of 0.5 hr. The suspension was stirred for an addition 0.5 hr, filtered and the residue washed with water. The residue was recrystallized from acetone-hexane to give 900 mg of 62 as colorless prisms: mp 129-130°; ir ($CHCl_3$) 2030 cm^{-1} (NCS), 1740, 1603 cm^{-1} (α-pyrone). λ_{max} (95% ethanol) 325 mμ (sh), 285 mμ (log ϵ = 4.40); nmr ($CDCl_3$) δ 2.4 ppm (d, 3, CH_3), 3.54 ppm (s, 3, OCH_3), 5.33 ppm (s, 2, methylenedioxy), 6.16 ppm (m, 1, olefinic proton), 7.25 ppm (AB, 2, J=9Hz, aromatic protons); mass spectrum molecular ion m/e 277.

Anal. Calcd. for $C_{13}H_{11}NO_4S$: C, 56.32; H, 4.00; N, 5.05. Found: C, 56.26; H, 3.96; N, 5.17.

8-[m-(Fluorosulfonyl)benzamido]-7-methoxymethyl-4-methylumbelliferone (65)

A solution of m-(fluorosulfonyl)benzoyl chloride (1 ml) in 20 ml of dimethylformamide was added dropwise to 8-amino-7-methoxymethyl-4-methylumbelliferone (500 mg) in 30 ml of dimethylformamide over 0.5 hr period. The solution was stirred an additional hour and then poured into 100 ml of water. The aqueous solution was extracted three times with 50 ml portions of chloroform. The chloroform extracts were dried ($MgSO_4$) and concentrated to dryness in vacuo. The residue was recrystallized from acetone to give 65 as white needles: mp 206-8°; ir ($CHCl_3$) 3430 cm^{-1} (N-H), 1737, 1620, 1580 cm^{-1} (α-pyrone), 1700 cm^{-1} (amide C=O); λ_{max} (95% ethanol) 325 mμ (inf), 315 mμ (log ϵ = 4.14), 305, 292, 282 mμ (sh); nmr (C_5D_5N)

δ 10.33 ppm (s, 1, NH), 8.3, 7.43 ppm (m, 6, aromatic protons), 6.16 ppm (m, 1, olefinic proton) 5.35 ppm (s, 2, methylenedioxy), 3.45 ppm (s, 3, OCH_3), 2.4 ppm (d, 2, CH_3); mass spectrum molecular ion m/e 421.

Anal. Calcd. for $C_{19}H_{16}NO_7SF$: C, 54.16; H, 3.83; N, 3.32. Found: C, 54.27; H, 3.90; N, 3.38.

8-[m-(Fluorosulfonyl)benzamido]—7-hydroxy-4-methylcoumarin (66)

A solution of 300 mg of 8-[m-(fluorosulfonyl)benzamido]-7-methoxymethyl-4-methylumbelliferone in 40 ml of acetone and 12 ml of 6N hydrochloric acid was stirred for 2 hr at room temperature. The aqueous solution was extracted three times with 20 ml portions of chloroform. The chloroform extracts were dried over magnesium sulfate, and concentrated to dryness under reduced pressure. Recrystallization of the residue from acetone gave 200 mg 66 as white plates: mp 248-258°; ir (KBr) 3315, 3240 cm^{-1} (O-H, N-H), 1700, 1600 cm^{-1} (α-pyrone), 1655 cm^{-1} (amide C=O); λ_{max} (95: ethanol) 320 mμ ($\log \epsilon = 4.18$) 281 mμ (sh), 252 mμ ($\log \epsilon = 3.91$); λ_{max} (1N, NaOH) 367 mμ ($\log \epsilon = 4.29$), 270 mμ ($\log \epsilon = 3.94$); λ^{F}_{max} (MeOH) 395 mμ; λ^{F}_{max} (pH 10) 460 mμ; nmr (D_6-DMSO)[Figure 7] δ 2.4 ppm (d, 2, CH_3), 6.18 ppm (m, 1, olefinic proton), 7.33 ppm (AB, 2, C_5 and C_6 protons), 8.33 ppm (m, 4, aromatic protons), 10.25 ppm (s, 1, N-H), 10.57 ppm (s, 1, O-H); mass spectrum molecular ion m/e 377.

Anal. Calcd. for $C_{17}H_{12}NO_6SF$: C, 54.12; H, 3.21; N,3.71.

Found: C, 53.98; H, 3.35; N, 3.80.

3-Substituted 7-Hydroxy-4-methylcoumarins

Ethyl-α-Oximinoacetoacetate (70)

Ethyl α-Oximinoacetoacetate was prepared by the method of Meyer (81) as modified by Albertson (82). A solution of ethyl acetoacetate (26.0 g) and sodium nitrite (13.8 g) in dilute potassium hydroxide (11.2 g/250 ml H_2O) gave 24.0 g yellow oil on acidification with sulfuric acid (6N). The crude product was dissolved in 30 ml of toluene, cooled to -15°C with stirring to give 20.2 g (64%) of 70: mp 55-6° [lit. (82) 58°]; ir ($CHCl_3$) 3550, 3220 cm^{-1} (O-H), 1740 cm^{-1} (ester C=O), 1700 cm^{-1} (ketone); nmr (CCl_4) δ 10.52 ppm (s, 1, O-H), 4.33 ppm (q, 2, J=7Hz, $O\underline{CH_2}CH_3$), 2.37 ppm (s, 3, $COCH_3$), 1.32 ppm (t, 3, J=7Hz, $OCH_2\underline{CH_3}$).

Ethyl α-Aminoacetoacetate hydrochloride (71)

Ethyl α-Aminoacetoacetate hydrochloride was prepared by the procedure of Gabriel and Posner (84) as modified by Laver and co-workers (83). Ethyl α-Oximinoacetoacetate (20.0 g) was reduced with stannous chloride (60.0 g) in concentrated hydrochloric acid (100 ml, 37%) to give 7.0 g (30.7%) of 71 as white prisms: mp 112-3° (dec) [lit. (83) 113-4° (dec)]; ir (nujol) 3000, 1575, 1515 cm^{-1} (NH_3^+), 1755 cm^{-1} (ester C=O), 1725 cm^{-1} (ketone).

3-Acetamido-7-hydroxy-4-methylcoumarin (74)

Condensation of resorcinol (2.20 g) and ethyl α-acetamido-

acetoacetate (3.20 g) in alcoholic hydrogen chloride (20 ml) by the procedure of Appel (57) gave 2.0 g of 74: mp 275-285°. Recrystallization of the crude product from ethanol-pyridine gave 1.6 g (40%) of 74 as white prisms: mp 283-5°; ir (KBr) 3325 cm^{-1} (O-H, N-H), 1720, 1635, 1610 cm^{-1} (α-pyrone), 1655 cm^{-1} (amide C=O); λ_{max} (MeOH) 324 mμ (log ϵ = 4.27), 254 mμ (log ϵ = 3.64), 243 mμ (log ϵ = 3.75); λ_{max} (1N NaOH) 375 mμ (log ϵ = 4.37), 235 mμ (log ϵ = 4.14); λ^{F}_{max} (MeOH) 400 mμ; λ^{F}_{max} (pH 10) 460 mμ; nmr (C_5D_5N [Figure 9] δ 10.33 ppm (s, 1, OH), 7.3 ppm (m, 3, aromatic protons), 2.4 ppm (s, 3, $COCH_3$), 2.29 ppm (s, 3, CH_3); mass spectrum molecular ion m/e 233.

Anal. Calcd. for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 61.93; H, 4.93; N, 6.05.

3-Amino-7-hydroxy-4-methylcoumarin (75)

3-Acetamido-7-hydroxy-4-methylcoumarin (200 mg) was refluxed in a mixture of concentrated sulfuric acid, glacial acetic acid and water (2:1:1 ml) for 2 hr, cooled and neutralized with dilute ammonia (2:1). The resulting precipitate was filtered and recrystallized from ethanol-water (1:1) to give 90 mg (55%) of 75 as white needles: mp 218-220°; ir (nujol) 3450, 3410, 3330, 3200 cm^{-1} (O-H, N-H), 1668, 1620 cm^{-1} (α-pyrone); λ_{max} (MeOH) 334 mμ (log ϵ = 4.26), 232 mμ (log ϵ = 4.09); λ_{max} (1N NaOH) 365 mμ (log ϵ = 4.20), 242 mμ (log ϵ = 4.25), nmr (C_5D_5N) [Figure 9] δ 7.2 ppm (m, 3, aromatic protons), 6.5-4.5 ppm (3, NH_2 and OH); 2.24 ppm (s, 3, CH_3).

Anal. Calcd. for $C_{10}H_9NO_3$: C, 62.82; H, 4.75; N, 7.33. Found: C, 62.76; H, 4.73; N, 7.43.

7-Hydroxy-3-isothiocyanato-4-methylcoumarin (76)

7-Hydroxy-3-isothiocyanato-4-methylcoumarin was prepared by the same procedure used to prepare 62. 3-Amino-7-hydroxy-4-methylcoumarin (200 mg) was added to a suspension of thiophosgene (460 mg, 0.30 ml) in a mixture of acetone (20 ml) and water (30 ml) to give 160 mg of 76 as light yellow prisms: mp 263-4°; ir (nujol) 3240 cm^{-1} (O-H), 2000 cm^{-1} (N=C=S), 1695, 1620, 1595 cm^{-1} (α-pyrone); λ_{max} (MeOH) 347 mμ (log ϵ = 4.45), 309, 254 mμ (inf), 237 mμ (log ϵ = 4.12); λ_{max} (1N NaOH) 395 mμ (log ϵ = 4.52), 245 mμ (log ϵ = 4.11); λ^{F}_{max} (MeOH) 470 mμ; λ^{F}_{max} (pH 10) 465 mμ; nmr (C_5D_5N) δ 8.0 ppm (1, O-H), 7.2 ppm (m, 3, aromatic protons), 2.25 ppm (s, 3, CH_3).

Anal. Calcd. for $C_{11}H_7NO_3S$: C, 56.66; H, 3.03; N, 6.01. Found: C, 56.64; H, 3.16; N, 6.09.

7-Hydroxy-4-methyl-3-(p-nitrophenyl)coumarin (77)

Diazotized p-nitroaniline (13.8 g) was coupled with 7-hydroxy-4-methylcoumarin (17.6 g) by the method of Sawhney and Seshadri (86) to give 8.0 g of 77 as light yellow cubes: mp 257-8° [lit. (86) 280-1°]; ir (nujol) 3300, 3200 cm^{-1} (O-H), 1680, 1615, 1600 cm^{-1} (α-pyrone), 1515, 1345 cm^{-1} (NO_2); λ_{max} (MeOH) 335 mμ (log ϵ = 4.36), 254 mμ (log ϵ = 4.10); λ_{max} (1N NaOH) 385 mμ (log ϵ = 4.40) 236 mμ (log ϵ = 4.26); nmr (D_6-DMSO) [Figure 11] δ 10.67 ppm (s, 1, OH),

8.31 ppm (d, 2, J=9Hz, aromatic protons), 7.66 ppm (m, 3, aromatic protons), 6.85 ppm (m, 2, aromatic protons), 2.23 ppm (s, 3, CH_3).

Anal. Calcd. for $C_{16}H_{11}NO_5$: C, 64.64; H, 3.73; N, 4.71. Found: C, 64.78; H, 3.77; N, 4.74.

3-(p-Aminophenyl)-7-hydroxy-4-methylcoumarin (78)

To a filtered solution of 3.18 g of stannous chloride in 25 ml of concentrated hydrochloric acid was added, portionwise and with stirring, 2.97 g of 7-hydroxy-4-methyl-3-(p-nitrophenyl)coumarin over 0.5 hr period. The mixture was stirred an additional 2.5 hr, filtered and residue washed with two 15 ml portions of water. The residue was dissolved in 10% sodium hydroxide, filtered and the filtrates neutralized with 4N hydrochloric acid with stirring. The resulting precipitate was filtered, washed with two 15 ml portions of water and dried to give 2.67 g (95%) of 78: mp 316-325° (dec); ir (nujol) 3360, 3300 cm^{-1} (O-H, N-H), 1682, 1620, 1608 cm^{-1} (α-pyrone); λ_{max} (MeOH) 331 mμ (log ϵ = 4.27), 243 mμ (log ϵ = 4.20); λ_{max} (1N NaOH) 370 mμ (log ϵ = 4.44), 294 mμ (log ϵ = 3.69), 249 mμ (log ϵ = 4.23); nmr (C_5D_5N) δ 7.35 ppm (m, 7, aromatic protons) 2.34 ppm (s, 3, CH_3); mass spectrum molecular ion m/e 267.

Anal. Calcd. for $C_{16}H_{13}NO_3$: C, 71.90; H, 4.90; N, 5.24. Found: C, 71.80; H, 4.95; N, 5.39.

7-Hydroxy-3-(p-isothiocyanatophenyl)-4-methylcoumarin (79)

Condensation of 700 mg of 3-(p-aminophenyl)-7-hydroxy-4-methylcoumarin with 600 mg (0.4 ml) of thiophosgene by the

procedure used to prepare 49 gave 425 mg of crude product. Recrystallization of the residue from acetone-hexane gave 370 mg of 79: mp 270-5° (dec); ir (nujol) 3210 cm^{-1} (O-H), 2190, 2120 cm^{-1} (N=C=S), 1675, 1595 cm^{-1} (α-pyrone); λ_{max} (MeOH) 328 mμ (log ϵ = 4.75) 282 mμ (log ϵ = 4.52); λ_{max} (1N NaOH) 370 mμ (log ϵ = 4.78), 272 mμ (log ϵ = 4.44); nmr (C_5D_5N) δ 9.0 ppm (s, 1, OH), 7.3 ppm (m, 7, aromatic protons), 2.16 ppm (s, 3, CH_3); mass spectrum molecular ion m/e 309.

Anal. Calcd. for $C_{17}H_{11}NO_3S$: C, 66.02; H, 3.59; N, 4.53. Found: C, 66.16; H, 3.67; N, 4.67.

Ethyl α-(p-Nitrobenzyl)acetoacetate (81)

From alkylation of acetoacetic ester (69) Ethyl α-(p-nitrobenzyl)-acetoacetate was prepared by the method of Clark and Johnson (88). A mixture of ethyl acetoacetate (69.0 g) and the sodium salt of ethyl acetoacetate (27.0 g) was treated with p-nitrobenzylbromide (37.3 g) to give 4.0 g of ethyl α,α-di-(p-nitrobenzyl)acetoacetate and 20.0 g ethyl α-(p-nitrobenzyl)acetoacetate.

From ethyl β-dimethylaminocrotonate (83) A solution of ethyl β-dimethylaminocrotonate (10.0 g) and p-nitrobenzyl bromide (17.3 g) in 50 ml of anhydrous methanol was refluxed for 5 hr. The solution was then allowed to stand for 12 hr at 25°. The methanol was removed under reduced pressure, 6 ml of water added to the oil and the mixture refluxed for 5 min. The residue was extracted with four 20 ml portions of

ether. The ethereal extract was dried ($MgSO_4$), concentrated, and distilled giving 14.0 (82%) of mono-alkylated product (81). The reaction product (81) had ir and nmr spectra identical with the compound prepared by Clark and Johnson (88): [lit. (88) 43°]; ir ($CHCl_3$) 1745 cm^{-1} (ester C=O), 1720 cm^{-1} (ketone), 1525, 1350 cm^{-1} (NO_2); nmr (CCl_4) δ 7.73 ppm (A_2B_2, 4, aromatic protons), 4.14 ppm (q, 2, OCH_2CH_3), 3.85 ppm (m, 1, methine), 3.23 ppm (2s, 2, benzylic protons), 2.13 ppm (2s, 3, CH_3CO), 1.2 ppm (t, 3, OCH_2CH_3).

Ethyl β-Dimethylaminocrotonate (83)

Ethyl β-dimethylaminocrotonate was prepared by the method of Glickman and Cope (89). Treatment of ethyl acetoacetate (60.0 g) with dimethylamine gas followed by distillation gave 36.0 g of 83: bp 79-81 (0.6 mm) [lit. (89) 121-2° (9.0 mm)]; ir ($CHCl_3$) 1680 cm^{-1} (C=O); nmr (neat) δ 4.47 ppm (s, 1, olefinic), 3.98 ppm (q, 2, OCH_2CH_3), 2.88 ppm (s, 6, $N-CH_3$), 2.38 ppm (s, 3, CH_3), 1.17 ppm (t, 3, OCH_2CH_3).

7-Hydroxy-4-methyl-3-(p-nitrobenzyl)coumarin (84)

By the sulfuric acid method Condensation of 1.65 g of resorcinol with 3.85 g of ethyl α-(p-nitrobenzyl)acetoacetate by the procedure used to prepare 44 gave 1.9 g of 84. The product (84) was crystallized from methanol: mp 283-285 (dec).

By the polyphosphoric acid method A mixture of 1.21 g of resorcinol, 2.8 g of ethyl α-(p-nitrobenzyl) acetoacetate

and 20 ml of polyphosphoric acid (115%) were heated gently on a steam bath. The temperature was kept in the range of 85-90° for 1.5 hr. The solution was cooled to room temperature and ice chips were added with stirring. The suspension was allowed to stand overnight, filtered and the residue was washed with water. Recrystallization from methanol gave 3.0 g (90%) of 7-hydroxy-4-methyl-3-(p-nitrobenzyl)coumarin. This material was identical with 84 prepared via the sulfuric acid method by comparison of mp and infrared spectra: mp 283-285 (dec); ir (nujol) 3300 cm^{-1} (O-H), 1675, 1600 cm^{-1} (α-pyrone), 1518, 1350 cm^{-1} (NO_2); λ_{max} (MeOH) 325 mμ (log ϵ = 4.34), 280 mμ (log ϵ = 4.13); λ_{max} (1N NaOH) 370 mμ (log ϵ = 4.38), 270 mμ (log ϵ = 4.10), 233 mμ (log ϵ = 4.20); nmr (C_5D_5N) [Figure 11] δ 10.22 ppm (s, 1, OH), 8.15 ppm (d, 2, J=9Hz , aromatic protons), 7.5 ppm (m, 3, aromatic protons), 7.1 ppm (m, 2, aromatic protons), 4.13 ppm (s, 2, benzylic protons), 2.33 ppm (s, 3, CH_3); mass spectrum molecular ion m/e 311.

Anal. Calcd. for $C_{17}H_{13}NO_5$: C, 65.59; H, 4.21; N, 4.50. Found: C, 65.70; H, 4.24; N, 4.68.

3-(p-Aminobenzyl)-7-hydroxy-4-methylcoumarin (85)

7-Hydroxy-4-methyl-3-(p-nitrobenzyl)coumarin (530 mg) was dissolved in 125 ml of boiling ethanol. To this solution

was added 230 mg of calcium chloride in 4 ml of water and 4.2 g of zinc dust. The mixture was refluxed for 4 hr and filtered while hot. The zinc cake was washed with two 15 ml portions of hot ethanol. The filtrates and wash solutions were concentrated under reduced pressure and the residue recrystallized from ethanol-pyridine to give 470 mg (55%) of 85 as tan prisms: mp 285-7° (dec); ir (nujol) 3385, 3305 cm^{-1} (N-H, O-H), 1680, 1600 cm^{-1} (α-pyrone); λ_{max} (MeOH) 323 mμ (log ϵ = 4.24), 237 mμ (log ϵ = 4.17); λ_{max} (1N NaOH) 365 mμ (log ϵ = 4.36), 233 mμ (log ϵ = 4.34); nmr (C_5D_5N) δ 7.2 ppm (m, 7, aromatic protons), 6.5-4.8 ppm (3, OH and NH_2), 4.01 ppm (s, 2, benzylic protons), 2.3 ppm (s, 3, CH_3); mass spectrum molecular ion m/e 281.

Anal. Calcd for $C_{17}H_{15}NO_3$: C, 72.58; H, 5.37; N, 4.98. Found: C, 72.55; H, 5.21; N, 5.01.

7-Hydroxy-3-(p-isothiocyanatobenzyl)-4-methylcoumarin (86)

7-Hydroxy-3-(p-isothiocyanatobenzyl)-4-methylcoumarin was prepared by the same procedure used to prepare 59. 3-(p-Aminobenzyl)-7-hydroxy-4-methylcoumarin (200 mg) was condensed with 328 mg (0.24 ml) of thiophosgene to give 200 mg (87%) of 86 as white prisms: mp 228-230°; ir (nujol) 3360 cm^{-1} (O-H), 2190, 2120 cm^{-1} (N=C=S), 1682, 1620, 1610 cm^{-1} (α-pyrone); λ_{max} (MeOH) 323 mμ (log ϵ = 4.61), 280 mμ (log ϵ = 4.64); λ_{max} (1N NaOH) 365 mμ (log ϵ = 4.66), 270 mμ (log ϵ =

4.54); nmr (C_5D_5N) δ 7.1 ppm (m, 7, aromatic protons), 4.02 ppm (s, 2, benzylic protons), 3.3 ppm (1, OH), 2.42 ppm (s, 3, CH_3); mass spectrum molecular ion m/e 323.

Anal. Calcd. for $C_{18}H_{13}NO_3S$: C, 66.87; H, 4.05; N, 4.33. Found: C, 67.00; H, 4.24; N, 4.45.

3-Substituted 7-Diethylamino-4-methylcoumarins

7-Diethylamino-4-methyl-3-nitrocoumarin (88)

In a 250 ml 3-necked, round-bottomed flask fitted with a mechanical stirrer, thermometer and dropping funnel was placed 20.0 g 7-diethylamino-4-methylcoumarin and 100 ml of sulfuric acid (d 1.80). The reaction flask was immersed in an ice-bath and a mixture of nitric acid (7.5 ml, d 1.42) and sulfuric acid (7.5 ml, d 1.80) was added slowly with stirring. The temperature of the solution was kept below 15°. After addition was complete, the stirred solution was allowed to warm to room temperature over a period of one hr. The mixture was poured over cracked ice with stirring, the aqueous solution was decanted and the solid residue washed with water and dried. The crude product was chromatographed on silica gel. Elution with benzene gave 8.0 g of 7-diethylamino-4-methyl-3-nitrocoumarin. Recrystallization of the crude product from benzene gave 6.6 g of 88 as yellow prisms: mp 164-6°; ir ($CHCl_3$) 1730, 1618, 1590 cm^{-1} (α-pyrone), 1527, 1352 cm^{-1} (NO_2); λ_{max} (95% ethanol) 420 mμ

(log ϵ = 4.41), 252 mμ (log ϵ = 4.21); nmr ($CDCl_3$) [Figure 14] δ7.51 ppm (d, 1, J=9Hz, C_5-H), 6.65 ppm (m, 2, C_6 and C_8 protons), 3.45 ppm (q, 4, $NCH_2\underline{CH_3}$), 2.44 ppm (s, 3, CH_3), 1.24 ppm (t, 6, $NCH_2\underline{CH_3}$).

Anal. Calcd for $C_{14}H_{16}N_2O_4$: C, 60.86; H, 5.84; N, 10.14. Found: C, 61.04; H, 5.70; N, 10.29.

3-Amino-7-diethylamino-4-methylcoumarin (89)

7-Diethylamino-4-methyl-3-nitrocoumarin (3.2 g) was added in small portions to a filtered solution of stannous chloride (11.0 g) in concentrated hydrochloric acid (30 cc). After addition was complete the solution was allowed to stir 4 hr (25°) during which time a white precipitate formed. The solution was allowed to stand for 2 hr and then filtered. The precipitate was dissolved in water, made basic with 10% sodium hydroxide solution and extracted with chloroform. The chloroform extracts were dried ($MgSO_4$), concentrated to dryness, and recrystallized from ethylacetate-hexane to give 2.33 g (76%) of 89 as light yellow prisms: mp 111-2°; ir ($CHCl_3$) 3490, 3400 cm^{-1} (N-H), 1700, 1630, 1595 cm^{-1} (α-pyrone); λ_{max} (95% ethanol) 378 mμ (log ϵ = 4.24), 259 mμ (log ϵ = 4.15); λ_{max} (1N HCl) 395 mμ (log ϵ = 3.38), 333 mμ (log ϵ = 4.20), 248 mμ (log ϵ = 3.86); nmr ($CDCl_3$) δ 7.32 ppm (m, 1, aromatic proton), 6.7 ppm (m, 2, aromatic protons), 3.7 ppm (2, NH_2), 3.4 ppm (q, 4, J=7Hz, $NCH_2\underline{CH_3}$), 2.18 ppm

(s, 3, CH_3), 1.2 ppm (t, 6, J=7Hz, $NCH_2\underline{CH_3}$); mass spectrum molecular ion m/e 246.

Anal. Calcd for $C_{14}H_{18}N_2O_2$: C, 68.30; H, 7.36; N, 11.38. Found: C, 68.36; H, 7.36; N, 11.46.

7-Diethylamino-3-isothiocyanato-4-methylcoumarin (90)

7-Diethylamino-3-isothiocyanato-4-methylcoumarin was prepared by the same procedure used for 62. 3-Amino-7-diethylamino-4-methylcoumarin (1.0 g) was condensed with thiophosgene (1.54 g, 1.0 ml) in 60 ml of acetone-water (1:1). The residue was recrystallized from ethyl acetate to give 1.08 g of 90 as fine yellow prisms: mp 161-2°; ir ($CHCl_3$) 2010 cm^{-1} (N=C=S), 1715, 1617, 1590 (α-pyrone); λ_{max} (95% ethanol) 412 mμ (log ε = 4.59), 264 mμ (log ε = 4.19); λ^{F}_{max} (MeOH) 490 mμ; nmr ($CDCl_3$) δ 7.32 ppm (d, 1, J=9Hz , C_5-H), 6.6 ppm (m, 1, C_6-H), 6.44 ppm (d, 1, J= 1.5 Hz, C_8-H), 3.41 ppm (q, 4, J=7Hz, $N\underline{CH_2}CH_3$), 2.35 ppm (s, 3, CH_3), 1.2 ppm (t, 6, J=7Hz, $NCH_2\underline{CH_3}$); mass spectrum molecular ion m/e 288.

Anal. Calcd for $C_{15}H_{16}N_2OS$: C, 62.49; H, 5.59; N, 9.72. Found: C, 62.64; H, 5.76; N, 9.80.

7-Diethylamino-3-[m-(fluorosulfonyl)benzamido]-4-methylcoumarin (91)

A solution of m-(fluorosulfonyl)benzoyl chloride (1.0 ml) in anhydrous acetone (15 ml) was added dropwise to a stirred solution of 3-amino-7-diethylamino-4-methylcoumarin

(325 mg) in anhydrous acetone (30 ml) over 15 min. The solution was stirred an additional 4 hr at 25°. During this time a yellow precipitate formed. The solid was dissolved by heating and allowed to crystallize slowly. A mixture of starting material hydrochloride and product was obtained. Recrystallization of the mixture from ethyl acetate gave 91 as yellow prisms: mp 189-191°; ir ($CHCl_3$) 3390, 3290 cm^{-1} (N-H), 1690 cm^{-1} (C=O), 1628, 1605 cm^{-1} (C=C), λ_{max} (MeOH) 380 mμ (log ε = 4.47), 249 mμ (log ε = 4.60); λ^{F}_{max} (MeOH) 470 mμ.

Anal. Calcd for $C_{21}H_{21}FNO_2O_5$: C, 58.32; H, 4.89; N, 6.48. Found: C, 58.36; H, 4.80; N, 6.61.

7-Diethylamino-4-methyl-3-(p-nitrophenyl)coumarin (92)

Diazotized p-nitroaniline (5.52 g) was coupled to 7-diethylamino-4-methylcoumarin (9.24 g) by the method of Long and Scalera (90) to give 4.5 g of 92 as orange plates from acetone: mp 176-8°; ir ($CHCl_3$) 1700, 1625, 1604 cm^{-1} (α-pyrone), 1525, 1352 cm^{-1} (NO_2); λ_{max} (MeOH) 395 mμ (log ε = 4.47), 248 mμ (log ε = 4.30); nmr ($CDCl_3$) [Figure 14] δ 8.26 ppm (d, 2, J=9Hz, aromatic protons), 7.5, 6.65 ppm (m, 5, aromatic protons), 3.46 ppm (q, 4, J=7Hz, NCH_2CH_3), 2.25 ppm (s, 3, CH_3), 1.22 ppm (t, 6, J=7Hz, NCH_2CH_3); mass spectrum molecular ion m/e 352.

Anal. Calcd for $C_{20}H_{20}N_2O_4$: C, 68.17; H, 5.72; N, 7.95. Found: C, 68.34; H, 5.86; N, 7.99.

3-(p-Aminophenyl)-7-diethylamino-4-methylcoumarin (93)

Reduction of 7-diethylamino-4-methyl-3-(p-nitrophenyl) coumarin (900 mg) with calcium chloride (221 mg) and zinc dust (6.66 g) by the procedure used for 85 gave 700 mg of 93 as orange prisms: mp 183-5°; ir ($CHCl_3$) 3500, 3415 cm^{-1} (N-H), 1705, 1625, 1595 cm^{-1} (α-pyrone); λ_{max} (MeOH) 380 mμ (log ϵ = 4.48), 303 mμ (log ϵ = 3.76), 255 mμ (log ϵ = 4.33); λ_{max} (1N HCl) 380 mμ (log ϵ = 3.72) 310 mμ (log ϵ = 4.09), 275 mμ (log ϵ = 4.15), 255 mμ (log ϵ = 4.23); nmr ($CDCl_3$) δ 7.0 ppm (m, 7, aromatic protons), 3.72 ppm (s, 2, NH_2), 3.42 ppm (q, 4, J=7Hz, $N\underline{CH_2}CH_3$), 2.24 ppm (s, 3, CH_3), 1.2 ppm (t, 6, J=7Hz, $NCH_2\underline{CH_3}$); mass spectrum molecular ion m/e 322.

Anal. Calcd. for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.46; H, 6.77; N, 8.51.

7-Diethylamino-3-(p-isothiocyanatophenyl)-4-methylcoumarin (94)

7-Diethylamino-3-(p-isothiocyanatophenyl)-4-methylcoumarin was prepared by the procedure used for 59, except in this case the solution was not refluxed. Condensation of 93 (500 mg) with thiophosgene (770 mg, 0.5 ml) in 40 ml of acetone gave 540 mg of residue. Recrystallization from acetone-hexane gave 500 mg of 94 as yellow prisms: mp 171-2°; ir ($CHCl_3$) 2190, 2120 cm^{-1} (N=C=S), 1695, 1610 cm^{-1} (α-pyrone); λ_{max} (MeOH) 385 mμ (log ϵ = 4.86), 278 mμ (log ϵ = 4.53), 248 mμ (log ϵ = 4.58); λ_{max}^{F} (MeOH) 470mμ; mass spectrum

molecular ion m/e 364.

Anal. Calcd. for $C_{21}H_{20}N_2O_2S$: C, 69.21; H, 5.53; N, 7.69. Found: C, 69.23; H, 5.38; N, 7.78.

7-Hydroxy and 7-Diethylaminocoumarin-4-acetic Acid and Derivatives

7-Diethylaminocoumarin-4-acetic acid (99)

7-Diethylaminocoumarin-4-acetic acid was prepared by the method of Kendall (96). Condensation of 10.0 g of dimethyl acetone dicarboxylate with 10.0 g of m-diethylaminophenol and 9.85 g of zinc chloride and subsequent hydrolysis gave 5.0 g of 99: mp 158-60° [lit. (96) 159-60°]; ir (nujol) 1715, 1670 cm^{-1} (C=O), 1600 cm^{-1} (C=C).

7-Hydroxycoumarin-4-acetic acid methyl ester (103)

7-Hydroxycoumarin-4-acetic acid methyl ester was prepared by the method of Burton (97). Condensation of 10.0 g of dimethyl acetone dicarboxylate with 7.4 g of resorcinol gave 7.0 g of colorless prisms when recrystallized from acetone: mp 208-9° [lit. (97) 209-10°]; ir (nujol) 3330 cm^{-1} (O-H), 1725 cm^{-1} (ester C=O), 1700, 1605 cm^{-1} (α-pyrone).

7-Hydroxy-3-(p-nitrophenyl)coumarin-4-acetic acid methyl ester (104)

Diazotized p-nitroaniline (13.8 g) was coupled with 103 (23.4 g) by the method used for 77. Recrystallization of reaction mixture from ethanol gave 6.0 g of 104 as light yellow prisms: mp 265-285° (dec); ir (nujol) 3160 cm^{-1}

(O-H), 1750 cm^{-1} (ester C=O), 1670, 1590 cm^{-1} (α-pyrone), 1520, 1345 cm^{-1} (NO_2); nmr (D_6-DMSO) δ 8.34 ppm (d, 2, J=9Hz, aromatic protons), 7.6, 6.8 ppm (m, 5, aromatic protons), 3.75 ppm (s, 2, CH_2-), 3.60 ppm (s, 3, OCH_3); mass spectrum molecular ion m/e 355.

Anal. Calcd for $C_{18}H_{13}NO_7$: C, 60.85; H, 3.68; N, 3.94. Found: C, 60.94; H, 3.57; N, 3.98.

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