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Mechanism of dansylation of the polyamine pentaazapentacosane · 5 HCl

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Abstract

Dansylation of the pentaamine pentaazapentacosane · 5 HCl (PAPC) produces only the perdansyl product. This occurs even under conditions of pH and dansyl chloride concentration most likely to produce partially dansylated products. This result is explained by a mechanism whereby only completely unionized amine molecules will dansylate. The proposed mechanism is supported by the dansylation versus pH profile of PAPC versus that of a reference monoamine (piperidine · HCl). After 4 h at room temperature and pH 9.5, 100% of piperidine is dansylated while under the same conditions only 10% of PAPC is derivatized. A pH greater than 10.5 is required to completely dansylate PAPC. This difference is significantly greater than would be predicted from the pK_a values but it is consistent with the proposed mechanism. © 1998 Elsevier Science B.V.

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1. Introduction

Pentaazapentacosane · 5 HCl (PAPC) is a synthetically produced polyamine which is being investigated for use as an anticancer agent (Fig. 1). PAPC does not absorb in the UV region and therefore, for most assays, it must be derivatized. Because dansyl chloride (1-dimethylamino-naphthalene-5-sulfonyl chloride, dns-Cl) is frequently used to increase either UV or fluorescent sensitivity of aliphatic polyamines (Seiler, 1975; Imai et al., 1984; Wilkinson, 1984) this was chosen as the derivatization agent.

The typical dansylation conditions for amines are pH 9–10, (Seiler, 1975; Imai et al., 1984; Wilkinson, 1984) and excess dns-Cl (Seiler, 1970) to push the reaction towards completion. Only the neutral form of the amine will react with dansyl

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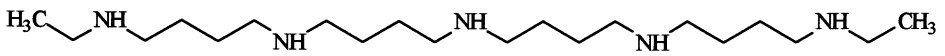

Fig. 1. Structure of pentaazapentacosane (PAPC).

chloride (Seiler, 1975), and therefore, the optimal pH must be high enough that the amine is primarily unionized. However, the pH is limited by an increase in side reactions, such as the formation of the sulfonic acid at high values (Gros and Labouesse, 1969).

The pK_a values for the five amine groups of PAPC range from 8.6 to 11.8. Therefore only some of the amine groups will be uncharged under the typical reaction conditions of pH 9–10 and partially dansylated products might be expected to be produced (Seiler, 1975; Peng et al., 1977). In fact, a total of 18 different partially dansylated compounds could conceivably be produced from this pentaamine. However, previous work has shown that only perdansyl PAPC, in which all potential reaction sites have been dansylated, is obtained under standard reactions conditions (Heimbecher et al., 1997). (Although the yield is decreased under unfavorable conditions of low pH and low dansyl chloride concentration only the pentadansylated derivative is produced.) The presence of partially dansylated products had not been demonstrated for any diamines or polyamines despite the many examples of their dansylation.

In this study, a mechanism is proposed which explains the perdansylation of PAPC based on the assumption that (i) only completely unionized amine molecules will dansylate and (ii) the ratio of unionized molecules to ionized molecules increases as dansylation proceeds. According to this mechanism, a significantly greater pH should be required to dansylate a polyamine versus a monoamine, and in a mixture of partially reacted compounds, the one with the highest degree of dansylation will react preferentially. This aspect of the mechanism is tested by comparing the dansylation versus pH curves for PAPC and the monoamine piperidine.

2. Materials and methods

2.1. Materials

PAPC was supplied by the National Cancer Institute (Bethesda, MD). Piperidine HCl, 99% (as the hydrochloride), was purchased from Aldrich (Milwaukee, WI). All other chemicals were at least reagent grade as described previously (Heimbecher et al., 1997).

2.2. Methods

2.2.1. pK_a determination

Solutions of 0.016 and 0.022 M PAPC (0.08 and 0.11 M as potassium hydroxide (KOH) equivalents) were titrated with 0.73 M KOH (which had previously been standardized with dried potassium acid phthalate). The water for all solutions was deionized and sparged with nitrogen gas before use. The titration set up included a Corning pH meter, Orion semimicro combination electrode and a Hamilton repeating dispenser. During the titration the tip of the dispenser was immersed in the solution and nitrogen gas was sparged over the surface. The titration curves were evaluated by calculating the average number of protons released from PAPC according to a spreadsheet method (Freiser, 1992). Linear regression lines were calculated with SAS (statistical analysis system).

2.2.2. Dansylation procedure

PAPC was derivatized with dns-Cl as described previously (Heimbecher et al., 1997). The derivatization conditions included a reaction solution of acetonitrile:aqueous triethylamine (TEA) pH adjusted buffer (60:40) which contained 14 nmoles PAPC/mL, 17 μmoles TEA/mL and 0.88 to 2.2 μmoles dns-Cl/mL. Before addition to the reac-

tion solution the buffer was adjusted to the appropriate pH. Samples were allowed to react for at least 4 h (no significant difference was seen between samples reacted for 4, 8 and 24 h).

The same conditions were used for the derivatization of piperidine except that only the 2.2 μmoles dns-Cl/mL concentration is used and the molar concentration of piperidine is five times higher than that used for PAPC. The higher piperidine concentration is to account for the fact that there are five derivatizable nitrogens per molecule of PAPC.

2.2.3. HPLC analysis

The dansylated samples were analyzed as described previously (Heimbecher et al., 1997). Briefly, this included use of a Beckman System Gold M406 liquid chromatograph and an adsorbosphere RP-C_8 column, 5 μm, 150 × 4.6 mm I.D. (Alltech Associates, Deerfield, IL). The HPLC conditions included a flow rate of 1.5 mL per minute, UV detection at 254 nm, injection volume of 100 μL and a mobile phase of 0.04 M pH 5 acetic acid:acetonitrile. The mobile phase was 45% acetonitrile for the piperidine assay and 90% acetonitrile for the PAPC assay.

2.2.4. Quantitation

The amount of dansylated PAPC detected is reported as percent relative recovery. This is calculated by assigning a value of 100% to the dansyl PAPC peak with the greatest peak area. The amount detected for all other PAPC samples is then calculated relative to this value. Similarly, the dansyl piperidine peak with the greatest peak area is assigned a value of 100% and the amount detected for all other piperidine samples is calculated relative to this value.

3. Results

3.1. PAPC pK_a values

The pK_a values for PAPC as determined by potentiometric titration are 8.6, 9.4, 10.3, 11.0 and 11.8. The 3.2 pK_a unit range covered by these values, is in line with ranges given for other polyionic compounds (Kimberly and Goldstein, 1981).

3.2. Perdansylation of PAPC

The dansylation procedure does not include an extraction step, and consequently, all of the dansylated products will appear on the HPLC chromatogram. The peak which eluted at 6 min, as shown in Fig. 2, was determined by mass spectrometry (Heimbecher et al., 1997) to be perdansyl PAPC (dns_5-PAPC). All other peaks on the chromatogram also appear in the blank. The additional peaks on the chromatogram were also further separated by adjusting the mobile phase. Again, except for the peak identified as perdansyl PAPC, all the sample chromatographic peaks also appear in the blank. The fact that no additional peaks were found when the chromatographic conditions were changed supports the conclusion that there are no partially dansylated PAPC products.

It should be noted that even under unfavorable dansylation conditions and short reaction times, no additional peaks were seen. These unfavorable conditions included derivatization at a low pH value of 8.5 and a low dansyl chloride concentration of 0.09 μmole/mL (Heimbecher et al., 1997). In addition, samples were assayed following short reaction times (zero time and 2 h). In all cases, no additional chromatographic peaks were found although the yield of the perdansyl PAPC substantially decreased.

3.3. Dansylation versus pH profile of PAPC

PAPC was dansylated under conditions of pH 8.5, 9.5, 10, 10.5 and 11 and dansyl chloride concentrations of 0.88, 1.3 and 2.2 μmole/mL (Heimbecher et al., 1997). As shown in Fig. 3 the recovery of PAPC generally increases with increasing pH. However, no further increase in dansyl PAPC was seen at pH values above 10.5.

3.4. Dansylation of PAPC vs. piperidine

The amount of dansylated product obtained for piperidine, which has a pK_a of 11.1 (Weast, 1972),

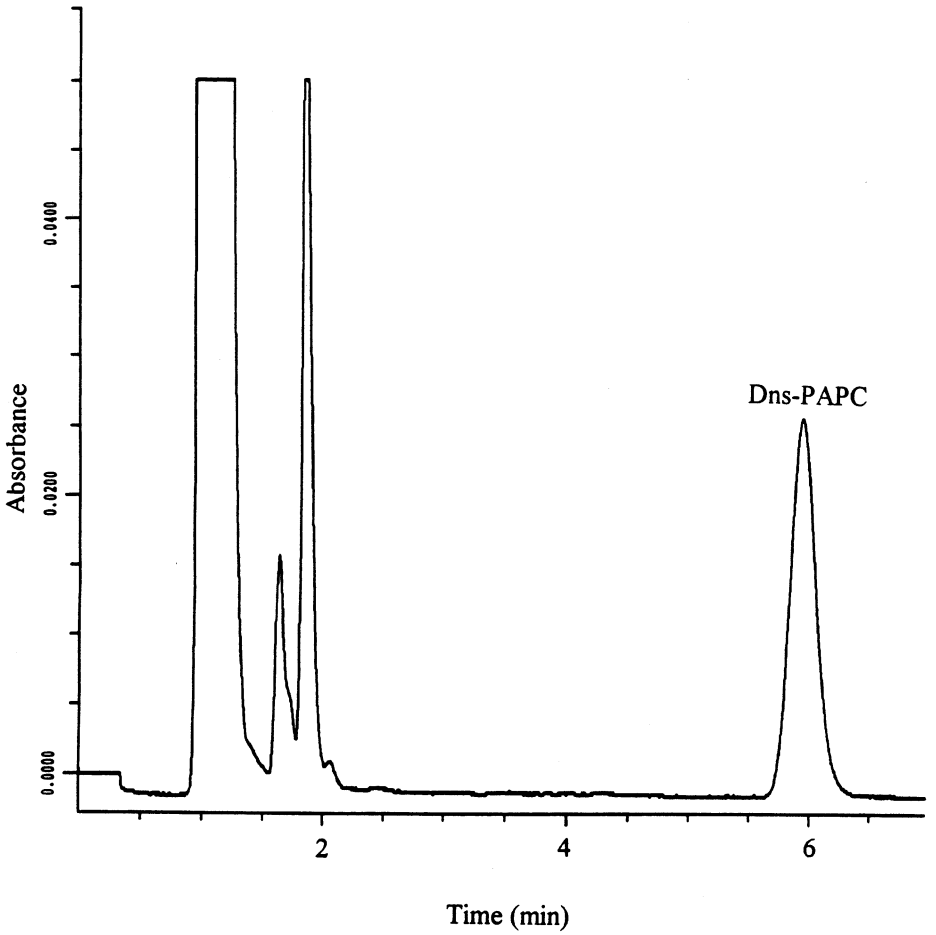

Fig. 2. Chromatogram of dansylated PAPC (Dns-PAPC). The amount injected was 0.3 nmole. The sample had been dansylated in a solution containing TEA buffer (pH 10.5) and 2.2 μmole dansyl chloride/mL. The peaks eluting before 2 min are by-products of the dansylation reaction, i.e. dansyl hydroxyl, dansyl glycine, dansyl amine and excess dansyl chloride.

was compared to that obtained for PAPC as a function of pH. Both the piperidine and the comparison PAPC samples contained 2.2 dns-Cl μmoles/mL and 70 nmoles of titratable nitrogen. As shown in Fig. 4, a much higher pH is required to achieve the same degree of PAPC dansylation as piperidine. For example, to get 80% dansylated product a pH of 10.0 is needed for PAPC while a pH of only 6.0 is sufficient for piperidine.

4. Discussion

The following mechanism of polyamine dansylation accounts for both the perdansylation of PAPC and the differences in the dansylation versus pH profiles for PAPC and piperidine. It is based on the premises that the polyamine molecule must be completely unionized before any one of the amine groups will react with dansyl chloride and secondly, that the fraction of uncharged molecules increases as dansylation progresses.

4.1. Requirement for unionized molecules

A possible reason for the first requirement is if dansyl chloride forms a positively charged intermediate, i.e. to the extent that derivatization takes place by an SN1 mechanism the sulfonyl group

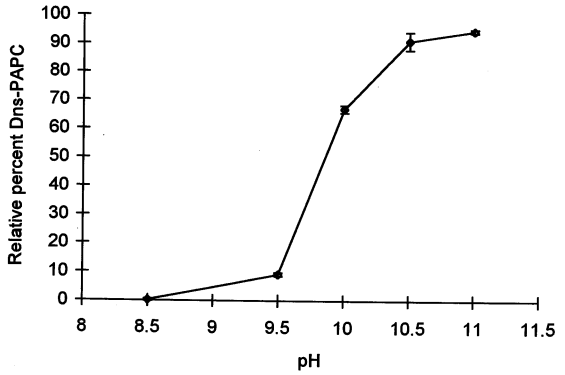

Fig. 3. Dns-PAPC versus pH. Mean ± S.D. for samples derivatized with 0.88, 1.3 or 2.2 μmole dansyl chloride/mL for 4, 8 or 24 h (◆, $n = 9$).

will carry a positive charge (Gordon et al., 1989). This positively, or partially positively charged intermediate would then be repulsed by any positive charges on the PAPC molecule. The repulsion could be due to direct affects by individual charged amine groups, or as suggested previously (King, 1965) any positive charges on the molecule could be distributed among all of the amine groups.

The requirement that the entire molecule be uncharged before dansylation will occur is crucial as this explains why no partial dansyl compounds are seen. Without this requirement each of the amine groups could dansylate 'independently', regardless of the ionization state of the other

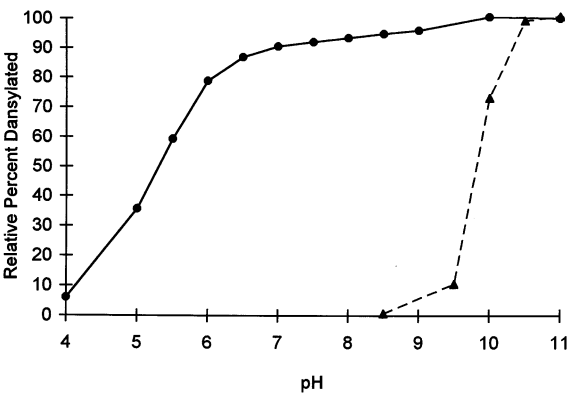

Fig. 4. Dansylation of Piperidine (●) and PAPC (▲). Sample concentrations of 70 nmoles piperidine/mL and 14 nmoles PAPC/mL were derivatized (as described in section 2.2.2.).

groups. For instance, PAPC has pK_a values of 8.6, 9.4, 10.3, 11.0 and 11.8. Therefore, at pH 8.5 about 50% of the PAPC molecules will have an unionized amine group and partially dansylated products should be formed. However, no partially dansylated compounds were found under this or any other condition.

On the other hand, if dansylation depends on the concentration of completely uncharged molecules little or no dansylated products would be expected at pH 8.5 where the fraction of uncharged molecules is only 1×10^{-7}. This is in agreement with the results. As shown in Fig. 3, no dansyl PAPC was detected at this pH. The requirement that all the amine groups be completely unionized could also explain the lack of partially dansylated compounds seen for other polyamines such as gentamycin (Peng et al., 1977).

4.2. Changes in the fraction of unionized molecules

The second assumption of the proposed model is that the fraction of uncharged molecules increases as dansylation progresses. If only the uncharged PAPC molecules will react, then the higher the fraction of uncharged molecules (f_u) the more the reaction will be driven towards dansylation. The f_u values, in turn, can be readily calculated for any compound from the dissociation constants (K_a) and the hydrogen ion concentration (Eq. (1)). In the case of a monoamine, such as piperidine,

$$f_u = K_a/([H^+] + K_a) \quad (1)$$

In the case of a pentaamine, such as PAPC, five protons must be lost to form the unionized compound and the fraction unionized (f_{u1}) is calculated by Eq. (2) (Freiser, 1992).

$$f_{u1} = \frac{K_{a1}K_{a2}K_{a3}K_{a4}K_{a5}}{[H^+]^5 + [H^+]^4K_{a1} + [H^+]^3K_{a1}K_{a2} + [H^+]^2K_{a1}K_{a2}K_{a3} + [H^+]K_{a1}K_{a2}K_{a3}K_{a4} + K_{a1}K_{a2}K_{a3}K_{a4}K_{a5}} \quad (2)$$

The constants K_{a1}, K_{a2}, K_{a3}, K_{a4} and K_{a5} are the five acid dissociation constants (K_{a1} is the effective K for dissociation of the penta-cationic compound, K_{a2} is the effective K for dissociation of

the three possible tetra-cationic compounds, K_{a3} is the effective K for dissociation of the six possible tri-cationic compounds, etc.). The concentration of PAPC in the unionized form is the product of the total PAPC concentration and the fraction which is unionized, i.e. $f_{u1} \cdot [PAPC]$. Similar equations can be used to calculate f_u values for each of the dansyl derivatives, i.e. monodansyl-PAPC (dns-PAPC), didansyl-PAPC (dns_2-PAPC), etc. For instance, for dns-PAPC the fraction unionized (f_{u2}) is calculated by:

$$f_{u2} = \frac{K'_{a1}K'_{a2}K'_{a3}K'_{a4}}{[H^+]^4 + [H^+]^3K'_{a1} + [H^+]^2K'_{a1}K'_{a2} + [H^+]^1K'_{a1}K'_{a2}K'_{a3} + K'_{a1}K'_{a2}K'_{a3}K'_{a4}} \quad (3)$$

where K'_{a1}, K'_{a2}, K'_{a3} and K'_{a4} are the four acid dissociation constants for monodansyl PAPC (Note that K'_{a1} is the effective K for dissociation of the tetra-cationic compound, etc.). The primary difference between f_u values for a pentaamine (Eq. (2)) versus a tetraamine (Eq. (3)) is due to the number of K_a values. For example, substituting any four, of PAPC's five K_a values, for K'_{a1}, K'_{a2}, K'_{a3} and K'_{a4} in Eq. (3), at any pH, gives $f_{u2} > f_{u1}$. This demonstrates that under all conditions the fraction of completely unionized molecules is inversely related to the number of similar ionizable groups on the molecule (assuming that the individual pK_a values do not increase with dansylation). The latter provision is discussed below.

4.3. pK_a values of PAPC derivatives

4.3.1. Electrostatic effects

The dissociation constants for PAPC and its derivatives are affected by a combination of electrostatic and statistical factors (Perrin et al., 1981). The pK_a values of PAPC dansyl derivative can be estimated by evaluating the effect that dansylation has on these factors. For PAPC, the electrostatic effect is mainly due to repulsion between positive charges on the molecule. Generally speaking the electrostatic lowering of the pK_a of an amine group is proportional to the number of positive charges on the molecule. Although the position of the charged amine groups will also affect dissociation, the primary effect results from the number of positive charges.

The first pK_a value for PAPC is quite low because, for the first dissociation, there are four positive charges that repel the proton of the fifth group. The second pK_a will be higher than the first because, following the first dissociation, there will be one less positive charge on the molecule. This trend continues until the highest pK_a, at which point no positive charges remain on the molecule and the electrostatic effect vanishes. (If the statistical effects are disregarded, the highest pK_a of a polyamine would be approximately the same as the pK_a of a structurally similar monoamine).

The electrostatic effect is also related to the number of positive charges on the dansyl derivatives of PAPC. For instance, dns-PAPC has four ionizable amine groups, and consequently, three positive charges are available to push the proton off of the first dissociating group. This is the same number of charges as are present for the second dissociation of PAPC and therefore, the electrostatic effects for these two species should be similar. Accordingly, if the statistical factors are ignored, and it is assumed that there are no specific interactions of the dissociating group with the dansyl moieties, the pK_a values for the first dissociating group of dns-PAPC and the second dissociating group of PAPC should be approximately equal. In a like manner, the pK_a values for the second dissociating group of dns-PAPC and the third dissociating group of PAPC should also be equal, and so forth.

4.3.2. Statistical effect

The statistical effect is based on the number of sites available for proton removal versus the number available for proton addition (Perrin et al., 1981). For example, for the fifth dissociation step of PAPC there is only one site for removing a hydrogen atom, but after this hydrogen is removed, there are five sites for adding a hydrogen, i.e. a 1:5 ratio. The effect this has on the pK_a will be log 5 or 0.7 pK_a units. This is in agreement with the experimentally found pK_a value of 11.8 which is 0.7 pK_a units higher than the pK_a value of a typical secondary amine which is 11.1 (Perrin et al., 1981).

Table 1
pK_a values for PAPC and its dansyl derivatives

Number of ionizable amine groups					
5	4	3	2	1	0
PAPC	dns-PAPC	dns_2-PAPC	dns_3-PAPC	dns_4-PAPC	dns_5-PAPC
8.6					
9.4	9.1				
10.3	10.1	9.8			
11.0	10.9	10.7	10.4		
11.8	11.7	11.6	11.4	11.1	none

Because the magnitude of the statistical effect depends on the number of ionizable amines, the statistical effect will decrease as dansylation proceeds and ionizable amines are converted to unionizable sulfonamides. For instance, after the first dansyl group is added there are only four ionizable amines and the difference between the first dissociation constant and the last, due to just this effect, would be calculated from (4:1)/(1:4), i.e. 16. Likewise, the ratio between the first and last dissociation constants of di, tri and tetra dansyl PAPC are 9, 4 and 1. Note that the statistical effect, like the electrostatic effect, vanishes for the pK_a of tetradansyl PAPC.

4.3.3. pK_a values for dansyl derivatives

Since the addition of each dansyl group decreases the number of potential positive charges by one, the first step in estimating pK_a values for dns-PAPC is to delete the lowest pK_a value of PAPC. Then, the changes in the remaining 4 pK_a values, (9.4, 10.3, 11.0 and 11.8), due to the statistical effect, i.e. going from 5 to 4 ionizable groups, is calculated. This involves first subtracting the statistical component from the pentaamine pK_a values and then adding in the statistical component for the monodansyl pK_a values.

For example, the statistical component of the second pK_a of PAPC is $-(\log 4/2)$ or -0.3 while the statistical component of the first pK_a of monodansyl PAPC is $-(\log 4/1)$ or -0.6 (Perrin et al., 1981). Therefore the pK_a of the first dissociating group for monodansyl PAPC is equal to 9.1, i.e. $9.4+0.3-0.6$. Starting with the estimated values for dns-PAPC, this procedure is then repeated to generate pK_a values for dns_2-PAPC, and these values are used to calculate pK_a values for dns_3-PAPC and so forth. The pK_a values generated in this way, for each of the partially dansylated compounds, are shown in Table 1.

4.4. Kinetic requirement for exclusive perdansylation

The reaction for formation of dns-PAPC is shown in Fig. 5. If the dns-Cl reagent is in excess and the reaction solution is buffered, the concentrations of dns-Cl and HCl are relatively constant and dansylation is pseudo first order. Dansylation of PAPC can now be represented by:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D \xrightarrow{k_4} E \xrightarrow{k_5} F$$

Scheme 1.

where $A = f_{u1} \cdot [\text{PAPC}]$, $B = f_{u2} \cdot [\text{dns}_1\text{-PAPC}]$ and $C = f_{u3} \cdot [\text{dns}_2\text{-PAPC}]$, etc. and k_1–k_5 are the rate constants for the uncharged species.

Scheme 1 can be further simplified to $A \overset{k_n}{\rightarrow} F$ since none of the partially dansylated compounds are produced in measurable quantities (Heimbecher et al., 1997). Therefore, the changes in concentration of *B*, *C*, *D* and *E* must be negligible relative to those of *A* and *F*, and the rate of formation of *F* is equal to the rate of loss of *A*.

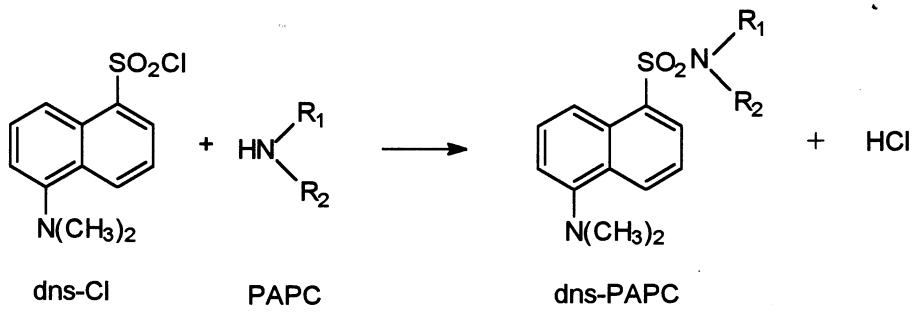

Fig. 5. Formation of a monodansyl PAPC.

This is consistent with a reaction scheme whereby $k_5 \gg k_4 \gg k_3 \gg k_2 \gg k_1$ (Connors, 1981) and dns_5-PAPC is the most energetically favorable derivative.

4.5. Rationale for perdansylation of PAPC

The basis for this stepwise increase in rate constants can be seen by examining the changes in the fraction of the various uncharged PAPC derivatives. As shown in Table 2, at any specific pH the f_u value increases as dansylation proceeds. Furthermore, because each f_u value is a constant at a constant pH, it can be combined with the corresponding rate constant shown in Scheme 1 to give an overall rate constant (k'). The k' values represent the rate constants in terms of the total concentration (charged and uncharged) for each species. For example the overall rate constant for formation of dns_1-PAPC is $k'_1 = k_1 \cdot f_{u1}$. Similarly, for dns_2-PAPC the overall rate constant is $k'_2 = k_2 \cdot f_{u2}$, and so forth (Connors, 1981).

If the k values are equivalent the k' values must increase progressively with dansylation, and $k'_5 \gg k'_4 \gg k'_3 \gg k'_2 \gg k'_1$. Therefore, the formation of dns-PAPC will facilitate the formation of dns_2-PAPC which in turn will facilitate the formation of dns_3-PAPC, etc.

4.6. Dansylation profiles of PAPC and piperidine

Fig. 4 shows the relative amounts of dansylated product obtained for PAPC and the monoamine piperidine as a function of pH. It is apparent that to achieve the same degree of dansylation PAPC requires a much higher pH than piperidine. However, this difference cannot be explained solely by the individual pK_a values for these two compounds. The dansylation of PAPC will be most limited by its highest pK_a value (11.8), and the difference between this value and the pK_a of piperidine is only log 5 or 0.7 pK_a units. Based on these values PAPC would require a pH only 0.7 units higher than piperidine to obtain equivalent amine dissociation and dansylation. It is therefore evident that the pH dependency of PAPC dansylation is not simply related to the concentration of uncharged amine groups.

Table 2
The fraction of unionized molecules (f_u)[a]

pH	PAPC (f_{u1})	dns-PAPC (f_{u2})	dns_2-PAPC (f_{u3})	dns_3-PAPC (f_{u4})	dns_4-PAPC (f_{u5})	piperidine (f_u)
9	1.7 E-7	8.5 E-7	6.3 E-6	1.5 E-4	7.9 E-3	7.9 E-3
10	4.4 E-4	9.9 E-4	2.7 E-3	1.2 E-2	7.3 E-2	7.3 E-2
11	6.7 E-2	9.5 E-2	1.4 E-1	2.4 E-1	4.4 E-1	4.4 E-1

[a]The f_u values are calculated by using equations of the form shown for Eqs. 1-3. The K_a values are calculated from pK_a 11.1 for piperidine and from the pK_a values shown in Table 1 for PAPC compounds.

All of these results are consistent with a mechanism by which dansylation, and its pH dependency, are related to the concentration of uncharged molecules. Table 2 shows the f_u values for PAPC, the dansyl PAPC's and piperidine at pH 9, 10 and 11. Note that since piperidine and dns_4-PAPC have one ionizable group and no additional charged groups they have the same f_u values at any pH. It can be seen that the difference between f_u values for undansylated PAPC and for piperidine is significant at pH 9 but diminishes as the pH is increased. Also, as the pH changes from 9 to 11 the changes in f_u for PAPC are much more dramatic than those seen for the piperidine.

These changes in f_u with pH are reflected in the dansylation of PAPC and piperidine. As shown in Fig. 4, a significant difference in dansylation is seen at low pH, and this difference diminishes as the pH is increased. The figure also shows that the dansylation versus pH curve is steeper for PAPC than for piperidine.

5. Conclusions

It is well known that for dansylation to occur, the amine group must be in the unionized form (Gros and Labouesse, 1969). Consequently, the effect of pH on dansylation is usually thought of in terms of individual amine groups. However, in this study it is shown that for a polyamine, such as PAPC, the ionization state of the entire molecule, and not just the amine group, is the key factor. The described mechanism requires an uncharged molecule for dansylation and predicts that formation of perdansyl PAPC is energetically favorable. This mechanism is consistent with both the complete lack of partially dansylated products and the pH/dansylation profile of PAPC and piperidine.

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