Enol esters as potential prodrugs. IV. Enhanced delivery of the quaternary species coralyne to rat brain using 6′-acetylpapaverin and its enol esters as prodrugs *

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Summary

Coralyne (I), 6′-acetylpapaverin (II) and three 6′-acetylpapverin enol esters (acetate, IIIa; isobutyrate, IIIb; and pivalate, IIIc) were compared for their ability to deliver the cytotoxic quaternary coralyne ion to the brains of rats following i.v. administration. Maximum levels of coralyne were achieved in all cases within one hour of dosing and remained essentially constant for up to 90 h, indicating an inability of the quaternary ion to escape from the brain. The brain levels achieved decreased in the following order; II > IIIa, IIIb > IIIc > I, with II and IIIa producing ~60- and ~30-fold greater brain coralyne levels than achieved when I was administered.

Based on the results obtained and previous work it appears that the determining factor in the accumulation of coralyne in brain following administration of the enol esters is the rate of production of II in the general circulation and not the rate of hydrolysis in brain tissue. Additionally, this study has demonstrated the utility of selected enol esters as prodrugs.

* For Part III of this series see refs., Repta and Patel (1981).

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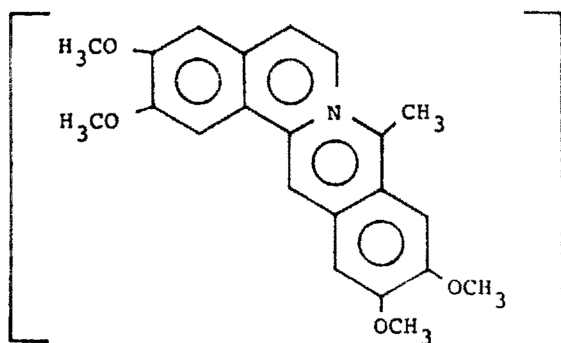
Introduction

Treatment of neoplastic and infectious diseases of the central nervous system (CNS) are hampered by the relative impermeability of the blood–brain barrier (BBB) to most chemotherapeutic agents. This inability to achieve therapeutic levels in the brain is basically due to the highly polar nature of the drugs and the lack of affinity for existing BBB transport systems, causing slow and incomplete penetration of the lipoidal barrier (Pardridge et al., 1975). However, it is possible, through various chemical modifications, to significantly alter a drug's permeability and distribution characteristics. This approach requires a quantitative reversion to the parent compound subsequent to fulfilling its delivery function. Several examples of such a prodrug approach with particular emphasis on BBB transport have appeared in the literature (Ross and Fröden, 1970; Bodor et al., 1976).

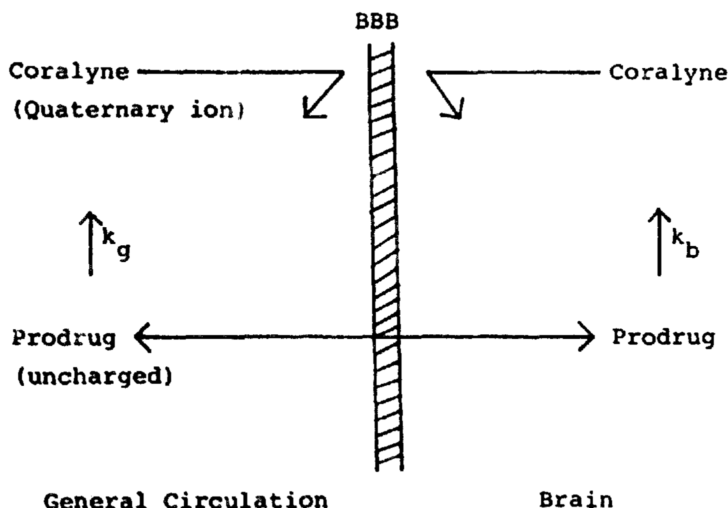


I

Coralyne

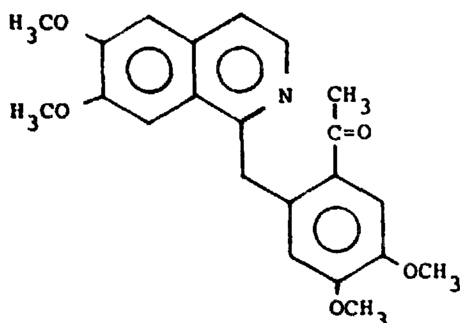
The experimental antineoplastic agent, coralyne (I), (as either the chloride or sulfoacetate salt), presumably due to the quaternary nature of the coralyne ion, is unable to achieve significant levels in the brain following both intravenous and intraperitoneal injections (Plowman et al., 1976). Distribution studies following administration of coralyne to mice and rats revealed that biliary excretion of the unchanged parent coralyne ion was its major route of elimination. Using [^{14}C]-radiolabelled coralyne, relatively high levels were found in the kidneys, liver and small intestine, while essentially no drug was found in the brain over the dosage range studied, i.e. 13–52 mg/kg.

Overcoming the observed inaccessability of coralyne to the brain compartment was the major objective of this work. Development of a prodrug system which might enhance the attainable levels of coralyne in brain tissues were based on identification of an uncharged precursor of coralyne which could pass the BBB and undergo reversion to the quaternary coralyne ion within the brain as shown in Scheme I, where k_g and k_b represent the rate constants for formation of coralyne from the prodrug in the general circulation and the brain, respectively. In such a scheme, several factors are potential contributors to elevated drug levels in the brain. These



include the enhanced permeability into the brain associated with the prodrug and the "trapping" of the quaternary coralyne in the brain due to its inability to permeate the BBB. In addition, the relative magnitudes of the rate constants, k_g and k_b, may also be expected to be important.

In earlier work Cho et al. (1975) and Schneider and Schroeter (1970) have shown that under highly alkaline conditions the coralyne ion undergoes ring opening to produce 6′-acetylpapaverin (II) (6′-AP), a neutral species which is potentially capa-



II

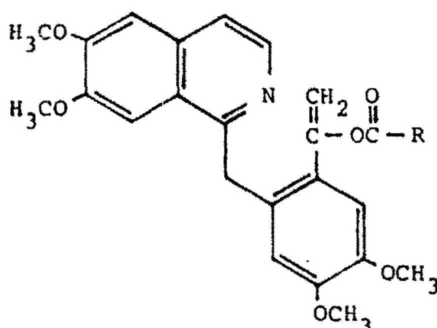
6'-Acetylpapaverin (6'-AP)

ble of traversing the BBB. 6′-AP was also shown (Cho et al., 1975) to rapidly undergo spontaneous recyclization to coralyne at physiological pH (pH = 7.4, 37°C, $t_{1/2} \sim 1$ min). While it is this spontaneous recyclization that makes the uncharged 6′-AP a potential prodrug of coralyne it is also this rapid cyclization that largely precludes its use because of formulation instability. Consequently, it appeared both necessary and desirable to modify the cyclization rate by formation of some derivative which could increase in vitro stability but when administered would revert in vivo to 6′-AP and ultimately to coralyne.

One approach could be chemical modification of the carbonyl functionality which is an enolizable ketone. Previously, we evaluated the overall potential of enol esters

as prodrug entities by studying the stability and enzyme-mediated hydrolysis of a model enol ester, α-acetoxystyrene (Patel and Repta, 1980). The results showed the enol ester to be a good substrate for endogenous esterases while also enhancing the aqueous stability. Further studies (Patel and Repta, 1981) concluded that careful selection of acyl groups of the α-acyloxystyrenes provided a means for alteration of both the stability in aqueous-buffered media and the rates of an enzyme-catalyzed hydrolysis.

Based on the analysis of the previous data obtained for the model α-acyloxystyrenes, 3 enol ester derivatives of 6′-AP were chosen to be evaluated as potential prodrugs of 6′-AP (Repta and Patel, 1981). The 3 esters chosen were 6′-AP enol acetate (6′-APA, IIIa), 6′-AP enol isobutyrate (6′-APIB, IIIb) and 6′-AP enol pivalate (6′-APP, IIIc). All 3 compounds demonstrated adequate aqueous stability to



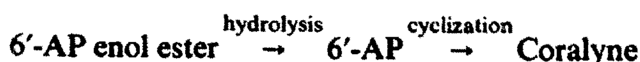
IIIa; 6'-acetylpapaverin enol acetate (6'-APA), R = CH_3

IIIb; 6'-acetylpapaverin enol isobutyrate (6'-APIB), R = $CH(CH_3)_2$

IIIc; 6'-acetylpapaverin enol pivalate (6'-APP), R = $C(CH_3)_3$

be potentially useful as prodrugs. The enol ester derivatives of 6′-AP also appeared to be adequate substrates for endogenous esterases of various tissue supernatants as well as human and rat plasma. The rates of hydrolysis in aqueous buffers and in biological media were substantially influenced by the structure of the enol ester, pH, plasma concentration and the nature of the tissues examined.

The rate of production and the distribution of coralyne in vivo following administration of an enol ester of 6′-AP could be expected to be a complex function of all those factors which affect distribution of the prodrug, rates of hydrolysis in the various biological fluids and tissues including the brain, and the rate of cyclization of 6′-AP. A greatly simplified form for the overall kinetic process is shown in Scheme II.



All the previous evidence tends to indicate general promise for the use of enol esters as prodrug candidates. More specifically, the chemical properties of the 6′-AP

enol ester strongly suggest that they may be useful in altering the distribution of coralyne to the brain. This work presents the findings of such in vivo studies.

Materials and methods

Materials

6′-AP was synthesized from coralyne (I) sulfoacetate (NSC 154890) (Supplied by the National Cancer Institute) according to the method of Schnieder and Schroeter (1920). The synthesis of the 6′-AP enol ester has been previously described (Repta and Patel, 1981). All the other reagents used were of analytical grade. Male Sprague–Dawley rats weighing 225–250 g were obtained from Harlan Sprague–Dawley, Madison, WI.

Rat studies

Solutions of 6′-AP, 6′-APA, 6′-APIB, 6′-APP and coralyne sulfoacetate were first prepared in dimethylsulfoxide (DMSO), because these compounds had low solubility in aqueous media. The DMSO solutions were subsequently diluted with a solution of dilute hydrochloric acid, pH ~ 2.2, to yield clear solutions. An acidic medium was used since the solubility of 6′-AP and its derivatives were expected to be greater under such conditions due to protonation of the isoquinoline nitrogen atom. The final solutions contained 10% v/v of DMSO in dilute hydrochloric acid solution. Coralyne sulfoacetate and the enol esters of 6′-AP had adequate stability in the formulation while solutions of 6′-AP itself were prepared just prior to use. The 6′-AP solutions were used within minutes after preparation to avoid cyclization of 6′-AP to coralyne prior to injection.

Coralyne sulfoacetate (7.5 mg), 6′-AP (5.7 mg), 6′-APA (6.31 mg), 6′-APIB (6.73 mg), and 6′-APP (6.94 mg) were each weighed separately and first dissolved in 0.5 ml of DMSO. Aqueous hydrochloric acid (4.5 ml, pH ~ 2.2 was added to each sample with gentle shaking to ensure complete dissolution of the compounds. Each of the resulting solutions contained the equivalent of 1.5 mg of coralyne sulfoacetate per ml of solution.

Solutions (~ 0.5 ml) of coralyne sulfoacetate, 6′-APA, 6′-APIB, 6′-APP and 6′-AP were administered into the exposed jugular vein of anesthetized (45 mg/kg of Nembutal sodium i.p., Abbott Labs.) rats over a period of 1–2 min. All rats, except those to be sacrificed one or two hours after injections, were surgically closed and allowed to recover from anesthesia. A solution of 70% ethyl alcohol in water was used as an antiseptic.

At designated times the rats were re-anesthetized (20–40 mg/kg) and sacrificed by infusing isotonic Sorenson's phosphate buffer (pH 7.4) into the heart to purge the blood from the brain quickly and completely (Munger et al., 1978). This infusion procedure helped remove any coralyne or coralyne precursors present in the brain capillaries. The brain, essentially devoid of all blood, was then removed, washed with cold isotonic Sorenson's phosphate buffer (pH 7.4) and stored in fresh buffer in a refrigerator until the tissue was analyzed for coralyne (within 24 h).

Determination of coralyne levels in brain tissue of rats

Fluorescence measurements were made with a Hitachi Perkin-Elmer Fluorescence Spectrophotometer (Model MPF2A). The following instrument settings were used for all measurements: excitation at $\lambda = 312$ nm with slit width of 9 nm; emission at $\lambda = 462$ nm with slit width of 16 nm, emission cut off filter at $\lambda = 430$ nm and a sensitivity setting of 2.

Extraction of coralyne from brain tissue

Rat brains, which had been removed and kept in cold (~5°C) buffer, were blotted dry and weighed. Each brain was homogenized using a 15-ml glass homogenizer with a teflon pestle (Series 3431-E04, A.H. Thomas, Philadelphia). The final volume of the homogenate was made to 12 ml with buffer solution.

An aliquot (5 ml) of that homogenate was placed in 15 ml screw-capped centrifuge tubes to which was added the ion-pairing agent, sodium heptafluorobutyrate (25 μl of 0.09 M aqueous solution), chloroform (7 ml) and sodium citrate (2 g). (The addition of citrate helped to salt out the proteins and increase the extraction efficiency of coralyne.) The centrifuge tube containing the homogenate, chloroform and citrate was then mixed using a vortex mixer until all the salt had dissolved. The resulting emulsion was subsequently centrifuged at $1000 \times g$ in a model FXD International Centrifuge (International Equipment, Boston). An aliquot (5 ml) of the bottom chloroform layer was removed and evaporated to dryness under a stream of nitrogen. Anhydrous methanol (10 ml) was then added to the residue. The resulting sample was capped to prevent loss of methanol and then placed in a model 220 Bransonic Ultrasonic bath (Bransonic Cleaning Equipment, Shelton, CT) for 10 min to assure complete dissolution of coralyne in methanol. The resulting methanol sample was turbid presumably due to precipitation of some proteins. Therefore, this sample was centrifuged again at $500 \times g$ for 10 min. The clear supernatant was diluted appropriately with methanol and its fluorescence measured.

Determination of extraction efficiency of coralyne from rat brain homogenates

The extraction efficiency of coralyne from rat brain homogenate was determined by extracting 5 ml of rat brain homogenate according to the procedure described above. The homogenate was prepared using one rat brain in 15 ml of isotonic Sorenson's phosphate, pH 7.4. The extraction efficiency was determined from homogenates containing ~35 ng and ~290 ng of coralyne sulfoacetate per ml and the mean values and standard deviations were found to be $83 \pm 2\%$ and $85 \pm 2\%$, respectively. Subsequently a recovery value of 85% was used to correct for the incomplete extraction of coralyne from the brain tissue.

Preparation of the standard curve

A stock solution of coralyne sulfoacetate was prepared in trifluoroethanol. Appropriate dilutions were made in anhydrous methanol, and a plot of the relative fluorescence vs concentration was made. The observed fluorescence was proportional to concentration in the range of 1–100 ng/ml of coralyne sulfoacetate in methanol. The linear expression of the data was $y = 0.891x + 0.903$ and the correlation coefficient for the line is > 0.999.

Results and discussion

Rats were administered 3 mg/kg coralyne sulfoacetate (CSA) or molar equivalent doses of either 6′-AP or one of the 3 enol esters (IIIa–c). After varying periods of time, up to 90 h, animals were sacrificed and the brains removed and homogenized. Aliquots of the homogenates were analyzed for coralyne and yielded the data shown in Fig. 1. Statistical analysis of these data by the Student-Newman-Keuls test (Sokal and Rohlf, 1969) confirmed the apparent rank-order of the various agents in delivery coralyne to the brain, i.e. 6′-AP > 6′-APA ~ 6′-APIB > 6′-APP > coralyne. This rank order persisted over the entire 8-h period [1].

From these data, it is clear that substantially higher levels of coralyne (I) in rat brains were achieved by administering 6′-AP and its enol esters than when CSA was used. The low levels of coralyne found in brain following administration of coralyne sulfoacetate salt are in agreement with those reported by Plowman et al. (1976). These low levels may be attributed to the fact that coralyne is a permanently charged species which normally experiences difficulty in passing through the BBB. When either 6′-AP, 6′-APA or 6′-APIB were administered, significantly enhanced levels of coralyne were found in the brain tissue. Also 6′-APP, while more effective than coralyne itself, produced much smaller increases than the other prodrug candidates.

The ~60-fold increase in coralyne levels effected by 6′-AP may be attributed both to the non-polar nature of the compound which allows it to permeate the brain as well as its relatively rapid rate of closure to yield coralyne. From these results it would appear that 6′-AP would be the best prodrug candidate. However, due to the instability of the compound (i.e. its rapid cyclization to coralyne in aqueous media) as previously reported (Cho et al., 1975; Repta and Patel, 1981), its pharmaceutical use is extremely questionable. The brain levels of coralyne found after i.v. administration of 6′-APA and 6′-APIB were about 30 × those achieved with coralyne itself, while 6′-APP produced a ~7-fold increase.

The increased levels of coralyne found in brain following the use of the prodrug forms may be achieved by eiter one or both of two alternative schemes. The first possibility is that the enol ester penetrates the BBB, and undergoes hydrolysis to 6′-AP which subsequently cyclizes to coralyne. The second possibility involves hydrolysis of the enol ester in tissue and fluids outside the brain. Some of the 6′-AP thus liberated could then permeate the BBB and undergo cyclization in the brain. The fact that 6′-AP yielded the highest brain levels of coralyne among the agents studied suggests that 6′-AP persists for some finite period of time in the general circulation and that it permeates the BBB and subsequently cyclizes to coralyne. The similarity of the effectiveness of both 6′-APIB and 6′-APA was somewhat surprising in view of previous in vitro results (Repta and Patel, 1981) showing that hydrolysis of 6′-APA in rat brain homogenate was considerably more rapid than the rate of hydrolysis of 6′-APIB. If the rate of hydrolysis in brain were the determining factor in the brain levels of coralyne achieved, it would have seemed likely that 6′-APA

[1] The 90-h values were not analyzed statistically but they appeared to maintain the same rank-order.

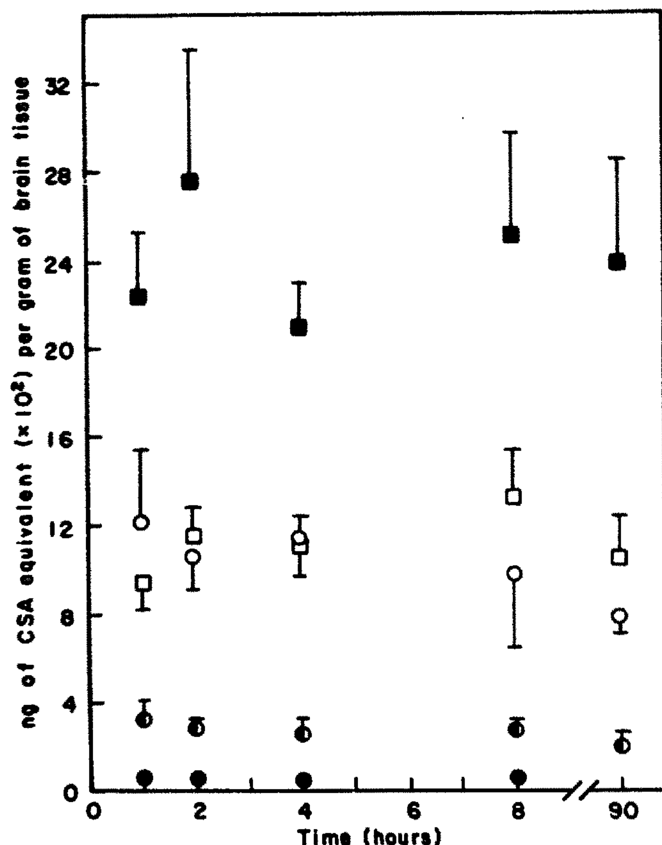


Fig. 1. Levels of coralyne found in the brains of rats following i.v. administration of 6′-AP (■), 6′-APA (○), 6′-APIB (□), 6′-APP (◐) and coralyne sulfoacetate (CSA) (●). For all agents the dose was equivalent to 3 mg of CSA/kg. Values at t = 1, 2, 4 and 8 h are means of 3 animals, while those at t = 90 h are from only two animals.

should have produced higher blood levels than 6′-APIB. However, the fact that 6′-AP itself produced such relatively high brain elvels of coralyne argues against the hypothesis that hydrolysis of the 6′-AP enol ester must occur in brain tissue in order to produce elevated coralyne levels in that organ. Furthermore, the previous in vitro studies (Repta and Patel, 1981) demonstrates that the highest hydrolytic activity toward the 6′-AP enol ester was found in liver and in this tissue both 6′-APA and 6′-APIB were hydrolyzed at very comparable rates. In total these data seem to suggest that the most important pathway contributing to the elevated brain levels achieved with the enol esters involves hydrolysis of the ester in the general circulation. The 6′-AP produced is then free to diffuse into the brain where it undergoes cyclization to coralyne.

The relatively low levels of coralyne observed after the administration of 6′-APP seem to support the above speculation since in in vitro studies (Repta and Patel, 1981) it was by far the most slowly hydrolyzed of the enol esters examined in all biological media studied. Although the above explanations do appear to agree with the data, other more complicated factors, i.e. altered distribution characteristics,

protein-binding, and enzymes associated with tissues and tissue fractions not considered in the earlier in vitro studies (Repta and Patel, 1981) cannot and should not be ruled out.

Another interesting finding of this study was that an analysis of variance (Sokal and Rohlf, 1969) showed, in the case of each agent used, that statistically there was no change in the levels of coralyne in brain over the period of 1–8 h. While the 90-h data consisted of only two animals and were not statistically analyzed, it appeared that little if any decrease in coralyne levels had occurred even after that time. Thus it appears that once coralyne enters brain tissue, its rate of efflux is quite slow. This finding is in general agreement with the proposed Scheme I where the quaternary species is unable (or slow) to cross the BBB. Furthermore, Ross and Fröden (1970) have shown that loss of quaternary species from brain tissue is exceedingly slow.

The fact that the brain levels remained essentially constant for 1–8 h also suggests that the delivery of coralyne by the various agents used is essentially completed in $\leq$ 1 h. If such were not the case, brain levels would plateau at later times. The completion of the delivery function might be expected to correspond to the loss of all prodrug species suggesting that total conversion to coralyne of all prodrug forms occurs in $\leq$ 1 h in vivo.

Previously, Plowman et al. (1976) demonstrated that administration of coralyne sulfoacetate to rats resulted in distribution of the drug in appreciable concentrations to all tissues except brain where the levels were very low. Those data suggest that coralyne is able to more rapidly penetrate non-brain tissues. Presumably, influx and efflux of coralyne into those tissues involves passive diffusion and thus as the drug is eliminated (~50% of dose was excreted at 96 h (Plowman et al., 1976)) the levels in the more readily accessible tissues would decrease proportionately. However, since coralyne in brain appears to be trapped in that tissue, the rate of decline would be expected to be much slower than in those other tissues which are accessible to coralyne. The net result of the situation would be an apparently selective enhancement of delivery of coralyne to brain. While there was no attempt made in the present work to assess levels of coralyne in tissues other than brain, the substantially greater fraction of the administered dose found in the brain as coralyne following the administration of 6′-AP, 6′-APA and 6′-APIB clearly demonstrates that enhanced delivery to brain tissue was achieved.

While the real efficiency of delivery of coralyne to brain in no case exceeded that expected if a uniform distribution of the drug to all tissues had occurred[2], the relative increases realized with the three most effective prodrug forms were impressive and significant.

Considering the non-linear pharmacokinetics and the protein binding of coralyne reported by Plowman et al. (1976), perhaps even greater selectivity of coralyne delivery to brain might be realized at other doses and dosage regimens.

[2] The dose of 3 mg/kg would produce a tissue level of 3 μg coralyne/g of tissue if rapidly and uniformly distributed to all tissues. Values obtained were about 2.6 μg coralyne with 6′-AP, while 6′-APA and 6′-APIB yielded ~1.2 μg coralyne/g.

Conclusions

In view of our findings it may be concluded that brain levels of the quaternary cytotoxic ion coralyne can be increased 30–60-fold by using non-quaternary prodrug forms of coralyne such as 6′-AP and some of its enol esters.

The fact that 6′-AP was the most effective prodrug delivery form of coralyne appears to suggest that the important in vivo kinetic step in the brain delivery of coralyne by the enol ester is the rate of hydrolysis in the general circulation rather than hydrolysis in the brain.

Although, 6′-AP was the most effective of the agents studied for enhancing the levels of coralyne in the brain, its instability in aqueous solutions (Cho et al., 1975) appears to preclude its use from a pharmaceutical standpoint. The solution stability problem of 6′-AP is largely obviated by use of its enol esters which are relatively stable (Repta and Patel, 1981) and potentially effective prodrugs of the coralyne ion.

More generally, this and preceding reports from these laboratories have clearly demonstrated that enol esters of enolizable carbonyl-containing compounds are useful derivatives for consideration as prodrugs.

Acknowledgemens

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