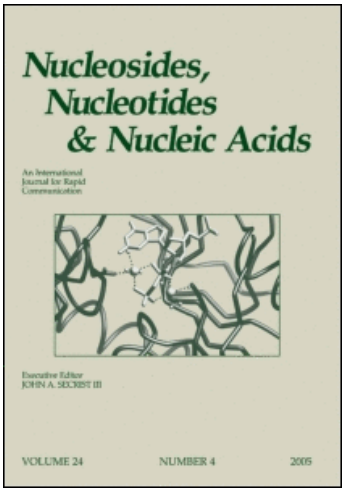

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Synthesis, Biochemical and Chemotherapeutic Activity of Some Azolo[1,3]Oxazine Nucleosides

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SYNTHESIS, BIOCHEMICAL AND CHEMOTHERAPEUTIC ACTIVITY OF SOME AZOLO[1,3]OXAZINE NUCLEOSIDES

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Summary: The synthesis of several 3,5,7-trisubstituted pyrazolo-[3,4-e][1,3]oxazines by ring annulation of the appropriately substituted pyrazoles is described. These specific compounds can be viewed as analogs of the C-nucleoside antibiotics formycin, formycin B and oxoformycin B. The biochemical and chemotherapeutic activity of these pyrazolo[3,4-e][1,3]oxazines is also disscussed.

A number of purines, purine nucleosides and purine-like nucleoside antibiotics (e.g., tubercidin (I), sangivamycin (II), toyocamycin (III), formycin (IV), formycin B, etc.) have exhibited[1-9] significant chemotherapeutic and biological activity. Numerous investigators, including our laboratory, have reported[1-9] on the results of their attempts to increase the chemotherapeutic and biological activity of these nucleosides by selective chemical modifications of the heterocyclic moieties. These modifications have included simple transpositions of atoms [e.g., purine vs pyrazolo[4,3-d]pyrimidine (formycin (IV) type nucleosides)], replacement by a methine group of a nitrogen atom [e.g., sangivamycin vs 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-d]pyrimidine-3-carboxamide (VI)], replacement of a nitrogen atom with a methine group [e.g., adenosine vs 3-deaza-adenosine (VII)], methylation of a ring nitrogen [e.g., formycin vs N-1- methylformycin], etc. These chemical modifications of active nucleosides have produced definite

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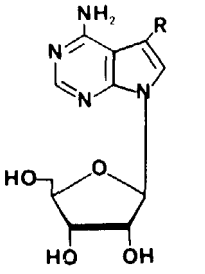
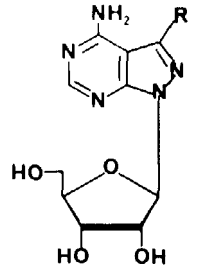
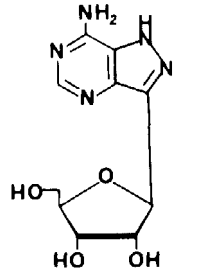
changes in their chemotherapeutic and biological activity. It has been reported[11,12] that the chemotherapeutic activity of formycin could be increased if this facile deamination could be prevented and this has been demonstrated by using the inhibitors coformycin[12,13] and deoxycoformycin[13]. We have also demonstrated that methylation of formycin to produce N-1-methylformycin[14] can effectively inhibit[13] the deamination of formycin without inhibiting the adenosine kinase reaction. The nucleoside formed[15] by the addition of a carboxamide group at the C-3 position of 4-APP-riboside[16] has not only prevented[17] enzymic deamination of the nucleoside by ADase, but also provided[17] a large increase in antileukemic activity (in vivo). The replacement of a ring nitrogen with a methine group [3-deaza-adenosine (VII)] has furnished[18] an adenosine analog which has been found[19] to function as neither a substrate nor an inhibitor of ADase. This nucleoside (VII) has also shown[20] some very interesting activity involving perturbations of certain biochemical



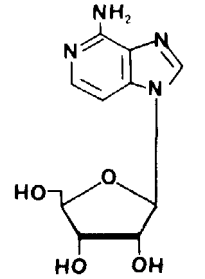
I, R = H
II, R = $CONH_2$
III, R = CN



V, R = H
VI, R = $C(=O)-NH_2$

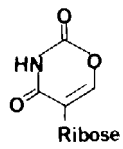


IV, (formycin)

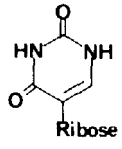


VII

transmethylations. There have been several reports[21] proposing that conformational aspects (syn vs anti) are of major importance in regards to whether adenosine analogs are substrates for ADase. However, we have recently shown[13] that in the case of formycin, although conformational aspects may be involved, they are not one of the major factors. In fact, it would appear that the major factors may be steric, electronic and/or the specific arrangement or juxtaposition of atoms in the heterocyclic moiety. We have strong evidence for the steric argument based on our N-methylformycin study and the 4-APP-riboside V vs nucleoside VI study[17]. This latter study is also of interest from the stand point of arrangement or juxtaposition of atoms in the heterocyclic moiety since 4-APP-riboside does not have a nitrogen at the purine seven position but can still function as a substrate for ADase which is in direct contrast to tubercidin (no purine N-7) which is not a substrate for ADase. This would suggest that electronic effects may be as important as the steric factor. One method for changing the electron density and distribution in the heterocyclic moiety is to attach specific exocyclic groups at certain positions of the ring system. However, this introduces the problem of trying to ascertain if the observed effect is due to electronic considerations or to a steric factor. To preclude this problem, we elected to insert an oxygen atom for a nitrogen atom in the pyrimidine ring of certain bicyclic compounds. This will produce a definite change in electron density of the heterocycle. This assumption is based on the change in pKa observed between uridine (pKa = 9.2) or pseudouridine (pKa = 9.0) and oxazinomycin (pKa = 6.96) where a ring nitrogen has been replaced by an oxygen atom. In some cases, there may also be a change in the hydrogen bonding capability as well as the electron density and distribution in the ring system of the resulting nucleoside. For our initial studies using this rationale, we elected to concentrate on the synthesis of pyrazolo[3,4-e][1,3]-oxazines. These specific compounds can be viewed as analogs of the C-nucleoside antibiotics formycin, formycin B and oxoformycin B.



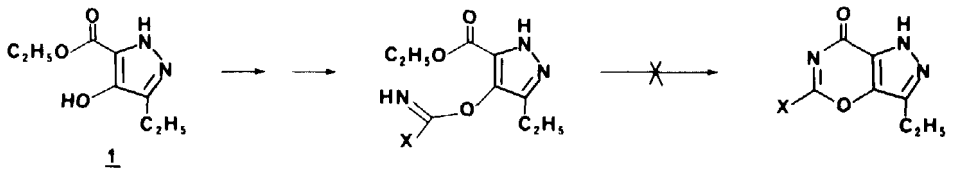
oxazinomycin

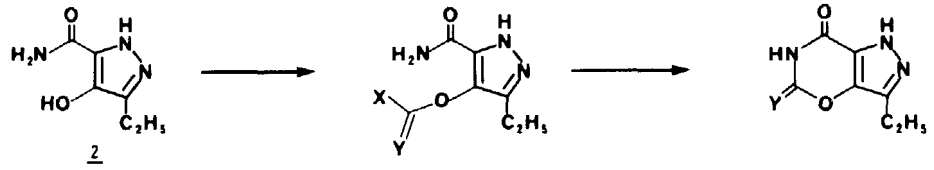


pseudouridine

Two approaches were investigated in our attempts to prepare this series of compounds. The first approach used 4-hydroxy-5(3)-ethyl-3(5)-carboethoxypyrazole (1)[22] as our starting material. Reactions of 1 with cyanogens or amidines should have resulted in a ring annulation by a nucleophilic attack of the imino group of the intermediate to the carboethoxy group.[23] However, there was no evidence of ring closure under our reaction conditions.

For our second approach we employed 3(5)-ethyl-4-hydroxypyrazole-5(3)-carboxamide (2)[22] as our starting material. Ring annulation of 2 using reagents such as bromocyanogen, chloroformamidine,[24] S-methylthiourea, etc., were unsuccessful. We subsequently found that ethyl chloroformate (ECF) in DMF containing triethylamine[25] would effect a ring annulation of 2 to furnish 3-ethylpyrazolo[3,4-e][1,3]oxazin-5,7-dione (3) in 45% yield. However, we were unable to obtain acceptable yields of other

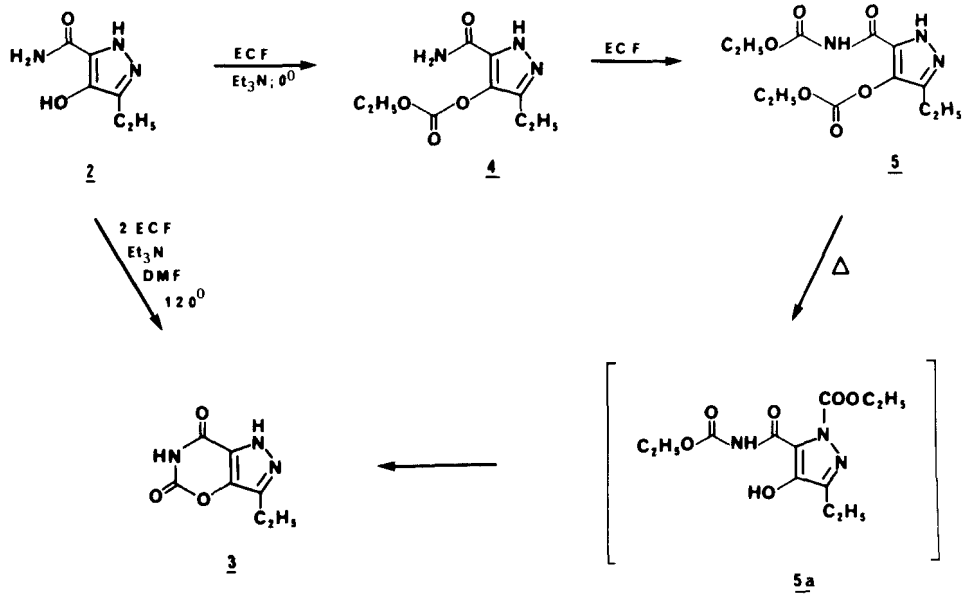




SCHEME 1

5,7-derivatives of 3 starting directly from 2. We presume that these difficulties are related to the tautomeric character of 2 and the facile O⇒N rearrangement of the intermediate O-derivatives as well as to the low stability of the newly formed C-O bond.

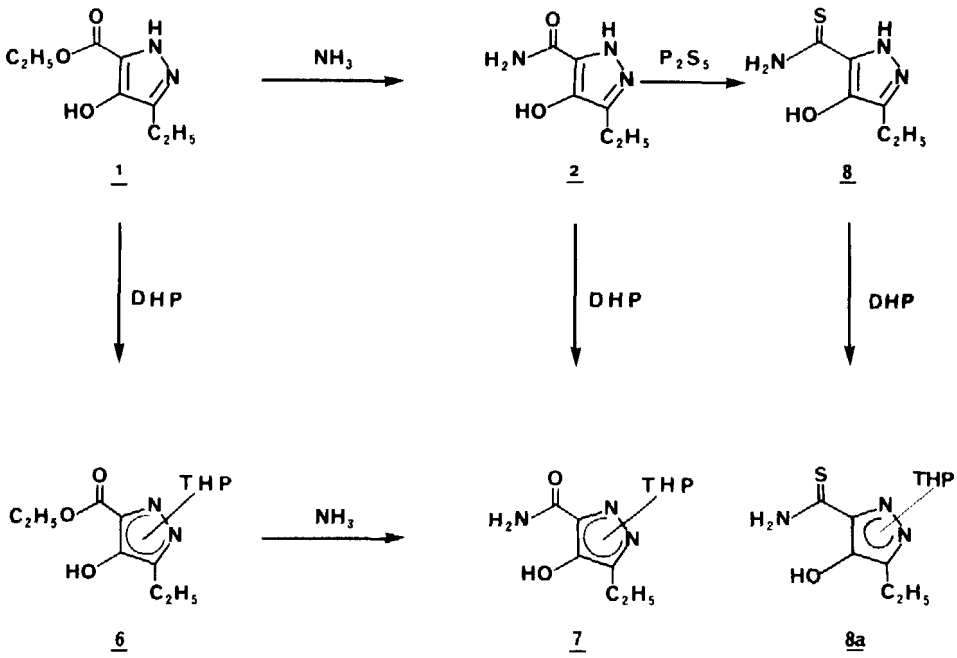
Treatment of 2 with ECF (ratio 1:1) furnished a product which we presumed was 4 since it gave a negative $FeCl_3$ test for a phenolic hydroxyl group and a strong peak in the IR spectrum at 1770 cm^{-1} and the following ^1H NMR spectral data in DMSO-d_6: δ, 13.1 (s, 1H, N_1-H); 7.08, 6.75 (br. d, 2H, $CONH_2$); 4.25 (q, 2H, OCH_2); 2.55 (q, 2H, CH_2); 1.25 (6H, CH_3). When an excess of ECF and triethylamine was used at 0-5°, a diacyl derivative (5) of 2 was obtained. The ^1H NMR spectrum of 5 in $CDCl_3$ showed two signals at δ 11.8 and 9.2 which were assigned to the N_1-H and CONHCO moieties, respectively, and signals at 4.25 (q, 4H, OCH_2), 2.65 (q, 2H, CH_2), 1.3 (m, 9H, CH_3) which supported the O-acylated structure 5. However, when the reaction was allowed to continue for several hours, a positive $FeCl_3$ test was again observed which indicated that a free hydroxyl group had been regenerated. A successful ring closure occurred when a twofold excess of ECF was added to the DMF solution of 2 and Et_3N at -10° followed by a gradual heating of the reaction mixture and then



SCHEME 2

heating at reflux for 2-3 hour. It would appear that some of the excess ECF serves as a protective group and is later removed during the work-up procedure.

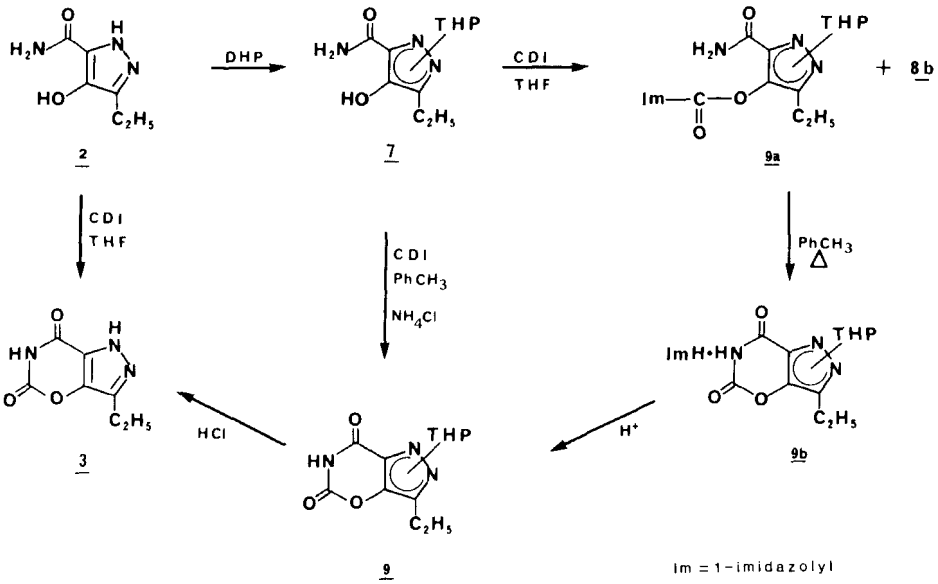
On this a priori assumption, we elected to block the N_1-position in an effort to avoid or reduce this type of complication and also to find a more facile and general way to effect the desired ring closing. In this context, we examined acylating agents which would provide a better leaving group as the protective group for blocking the N_1position. Selective acetylation of pyrazofurin was previously reported,[26], but this protecting group is unstable under the basic conditions required for the ultimate ring closing reaction. 2,3-Dihydropyran (DHP) was the reagent we finally selected for protection of the pyrazole ring because of its apparent selectivity with OH and NH nucleophiles,[27-29] stability under basic conditions and lability under very mild acid conditions.[28,30] 1,1'-Carbonyldiimidazole (CDI) and the corresponding thioanalog (TCDI), which have been successfully employed for various ring closures,[31,33] were selected as the acylating reagents, for our initial studies in this area.



1 2 8 6 7 8a

SCHEME 3

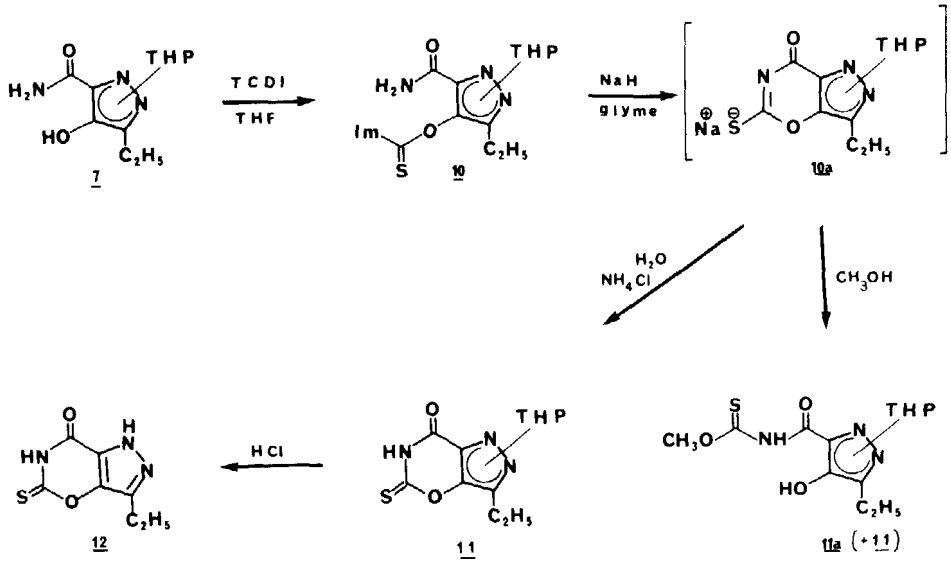
Even though there are two nucleophilic centers (in 1) or even three (in 2 and 8), we found that the pyrazole ring of these compounds may be selectively protected by DHP under mild acid catalysis. The protected derivative 6, 7, 8a have been obtained using slightly modified literature procedures.[28,30] Compounds 6, 7 and 8a showed a positive reaction with $FeCl_3$ as well as the correct IR, ^1H NMR and MS spectra. In contrast to some reported[43] THP-protected purines (obtained as single isomers), 6 and 7 were obtained as mixtures of two isomers as ascertained by TLC and ^1H NMR. Two single isomers of 6 (6a and 6b) were separated by column chromatography (SiO_2, $CHCl_3$). Each isomer was chromatographically pure by TLC and although they gave essentially the same elemental analysis they possessed different mp and spectral data (UV, ^1H NMR, ^{13}C NMR). One of the isomers showed a substantial downfield chemical shift for the 2'-proton signal ($\Delta\ \delta$ = 0.57 ppm). This downfield shift is related to the deshielding effect of an adjacent carboethoxy group and allowed us to make structural assignments to both isomers. According to previous studies,[34,35] thiocarboxamido and carboxamido groups may deshield a neighboring anomeric proton ($\Delta\ \delta$ = 0.2 to 0.4 ppm) due to an anisotropic



SCHEME 4

effect. The two isomers also exhibit different patterns for the C-3, C-4 and C-5 atoms in the ^{13}C NMR spectra. This has proved to be a very useful tool for determining the position of the THP group during further transformations and in other structurally similar compounds. However, for our purpose, the isomeric mixture was used for subsequent reactions without effecting a separation of the isomers.

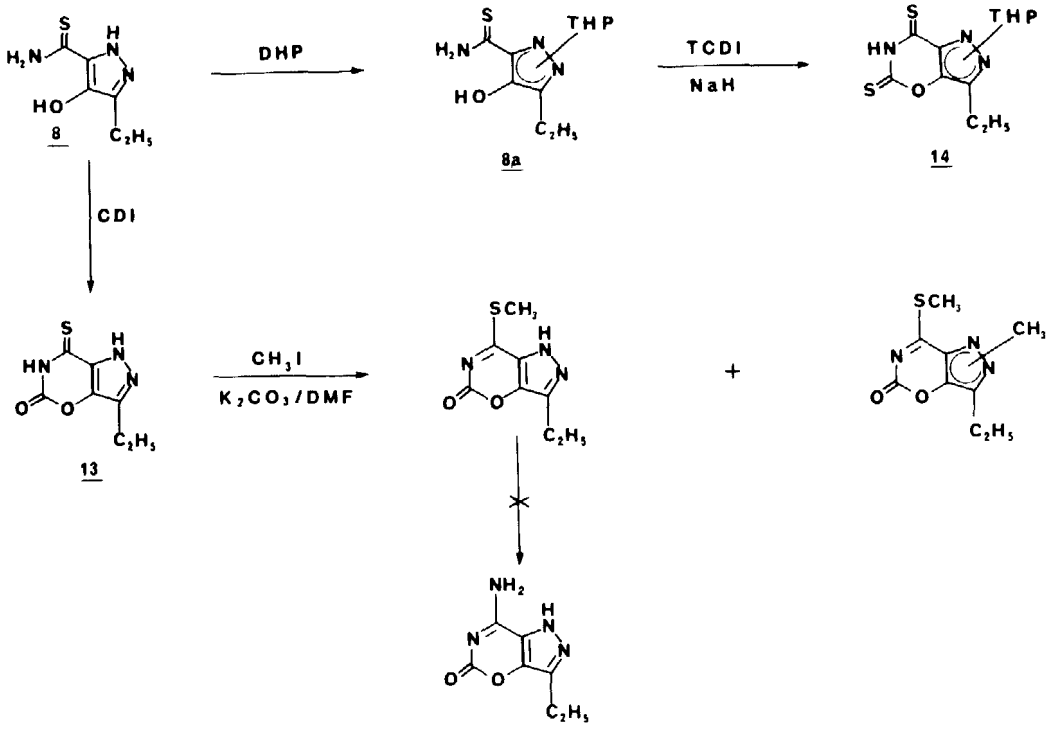
Unlike ECF, CDI reacted very readily with 2 and 7 in aprotic solvents at reflux (Scheme 4). Compound 9a was the first product formed in the reaction with 7. The ^{1}H NMR spectrum of 9a, isolated from the mixture (9a + 9b) by column chromatography, showed three signals for the imidazole ring protons at 8.45, 7.5 and 7.15 ppm as well as signals assigned to an amino group at 7.28 and 7.50 ppm. However, the spectrum of 9b revealed only two signals for the aromatic protons of the imidazole cation at 7.10 (s, 2H, C^{5},$_{4}$-H) and at 7.72 (s, 1H, C_{2}-H). Treatment of 9b with NH_4OH was followed by treatment with a cation exchange resin (Amberlite, IR-120, H^{+}) to precipitate pure 9. We subsequently found that a reaction of 7 with CDI in toluene with the use of ammonium chloride in the work up gave a 45% yield of 9. Deprotection of 9 fur- nished compound 3 which was identical to 3 obtained directly from 2.



SCHEME 5

In contrast to CDI, the reaction of 1,1'-thiocarbonyldiimidazole (TCDI) with 2 gave a complicated mixture of compounds. Reaction of TCDI with 7 in THF at reflux furnished compound 10 as the only major product. Increasing the temperature of the reaction mixture either did not result in ring closing or gave complicated mixtures[31]. Sodium hydride has been used to increase the nucleophilicity of the carboxamide group and has effected a ring closure of 10 to 11. A solution containing 10 in glyme with NaH was heated at reflux to furnish 11 as a major product. A problem which we had anticipated, and indeed encountered in this study, was the instability of the oxazine ring which required special attention. Using a methanol-water solution for our work-up, resulted in a ring opening and gave a mixture of 11 and 11a. A singlet of δ 4.2 in the ^{1}H NMR spectrum was assigned to the methoxy protons and signals at 9.8 and 7.1 (OH, NH) provided additional proof for the proposed structure of 11a. The mass spectrum showed a parent ion of m/z 313 and a characteristic pattern for the open-chain structure fragmentation. (We also used the classical test with $FeCl_3$ to verify ring closure or integrity at each synthetic step). Compound 11 was obtained by taking up the solid residue (10a), after glyme evaporation, in chloroform followed by treatment with a cold saturated NH_4Cl/H_2O solution. The ^{1}H NMR spectrum of 11, purified by column chromatography, revealed a broad singlet at 9.98 (NH) and characteristic signals for the THP and ethyl groups. A high value for the carbonyl absorption (1720 cm^{-1}) in the IR spectrum of 13 is also very characteristic and may be the result of a competition for the unshared electron pair of the ring nitrogen (N_6) between the carbonyl and thiocarbonyl groups.[36] Deblocking of 11 furnished 3-ethylpyrazolo[3,4-e][1,3]oxazin-7-one-5-thione (12).

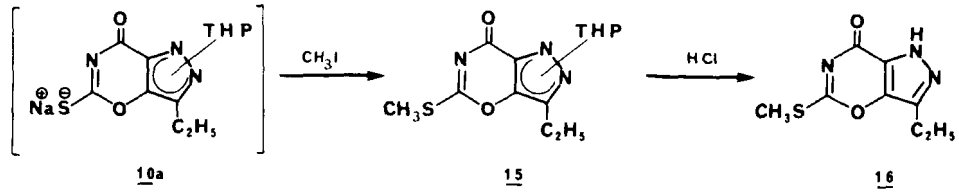
When the thioamide compound 8 was allowed to react with CDI in THF at reflux, the bicyclic compound 13 was obtained in approximately 80% yield. The reaction of 8 with TCDI proved to be as unsuccessful as when TCDI was reacted with 2. However, the protected thioamide 8a reacted very readily with TCDI and furnished 3-ethyl-



SCHEME 6

pyrazolo[3,4-e][1,3]oxazin-5,7-dithione (14). Methylation of 13 with methyl iodide furnished a mixture of the mono- and dimethyl derivatives of 13.

Exocyclic methylthio group have been found to function very effectively as leaving groups in the purine series.[37] It was also observed that their reactivity is dependent on the position as well as on the heteroaromatic character of the fused bicyclic heterocycle in the case of some structurally related purine analogs.[37] In our case, we expected to observe a facile displacement of the methythio-group because of the strong -I effect of the adjacent ring oxygen atom. This prompted us to prepare the appropriate methylthio derivative as a precursor for the guanine analog. Direct methylation of the sodium salt 10a with CH_3I furnished the methylthio derivative 15 in 54% yield. Subsequent deblocking using our standard procedure gave 3-ethyl-5-methylthiopyrazolo[5,4-e][1,3]oxazin-7-one (16). Aminolysis (displacemnt of a methylthio group) is usually

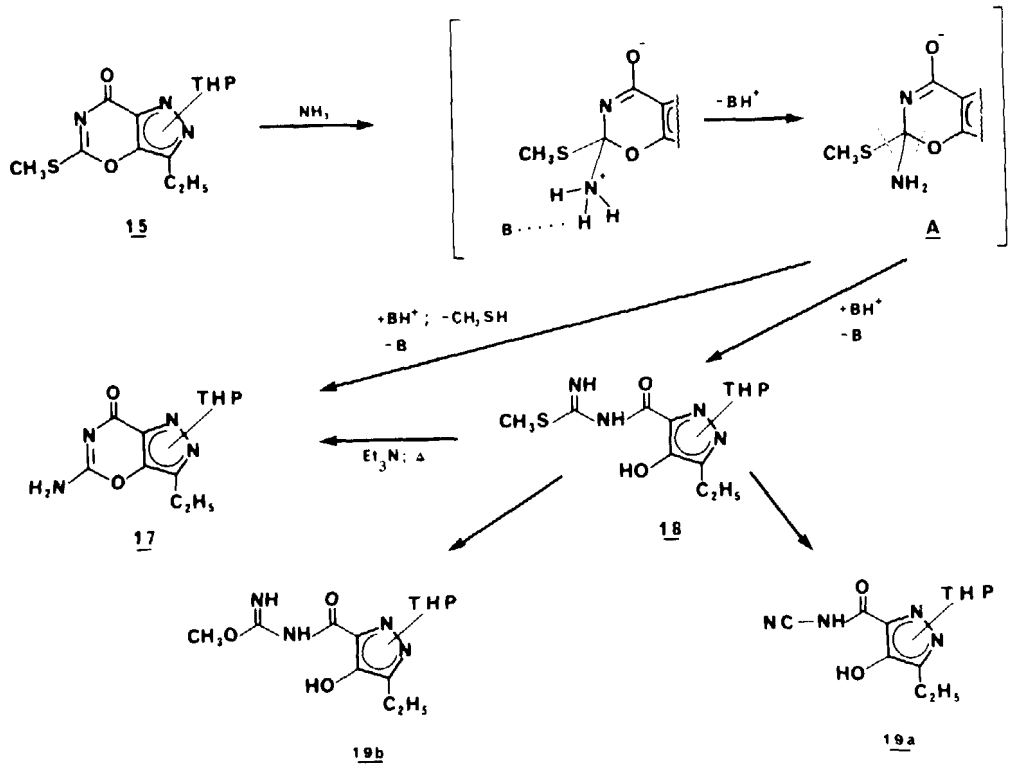


10a 15 16

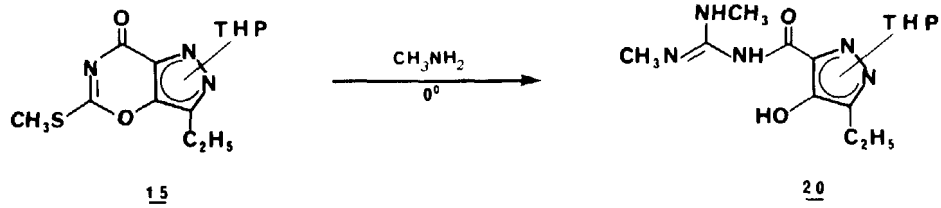
SCHEME 7

accomplished by using either liquid ammonia or a methanol ammonia solution. Treatment of 15 by either method gave complicated reaction mixtures. These mixtures showed a positive $FeCl_3$ test which would indicate that some ring opening had taken place. Some of the side-products from these mixtures were separated and analyzed (Scheme 8).

Compound 19a was obtained from the reaction of 15 with liquid ammonia at -50° and revealed a strong absorption at 2160 cm^{-1} (C ≡ N) with a characteristic low value for the carbonyl absorption 1620-1600 cm^{-1}). There were no signals observed in the NMR spectrum of 19a which could be attributed to a methylthio group, however, when the reaction was conducted in methanol or a methanolmethylenechloride mixture compound, 19b was the major side-product. The ^1H NMR spectrum 19b revealed a signal at δ 3.9 which could be attributed to a CH_3O group and a broad absorption at δ 9.8 - 9.2 which was attributed to NH and OH hydrogens. When the reaction was conducted in methylene chloride, compound 18 was the major side-product. The ^1H NMR spectrum of 18 showed a sharp absorption at δ 2.5 which could be attributed to the SCH_3 group and a broad absorption at δ 9.5 - 9.0 (NH, OH). The mass spectra of 19b and 18 gave peaks for the molecular ions at 296 and 312 m/z, respectively. According to the assumed mechanism for this reaction, the nucleophilic substitution was accompanied by subsequent ring opening (a competition between the two leaving groups takes place in A.) Therefore, two mechanisms S_N(AE) and ANRORC may be involved in this reaction.[38,39] When compound 18 was dissolved in benzene and the solution heated at reflux in the presence of Et_3N, TLC showed a slow transformation of 18 into 17.

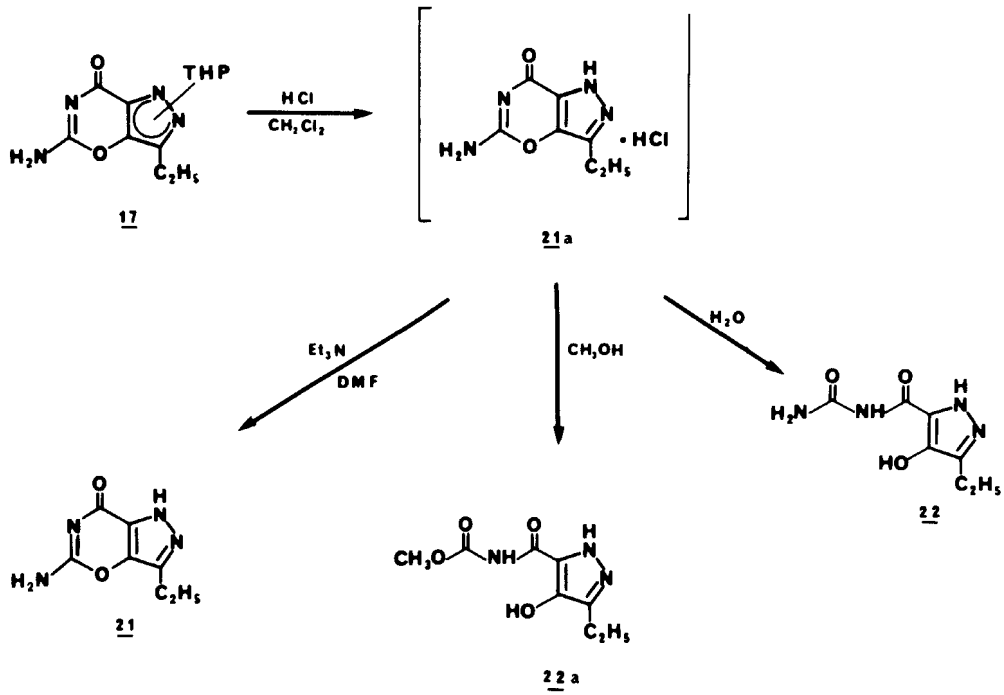


SCHEME 8



SCHEME 9

After this preliminary study, the amination of 15 was conducted as a two step reaction in dry methylene chloride containing Et_3N. The first step involved passing NH_3 (gas) through the reaction mixture at 10° and the sealed flask was then allowed to stand at room temperature until we observed a complete disappearance of 15. The second step was to heat the reaction mixture at reflux for 4 hours in order to convert 18 into 17. Longer reaction times usually resulted in excessive decomposition. This procedure allowed us to simplify the work-up and purification process which increased the



SCHEME 10

yield of 17 from 15-20% to 65%. The only product we could isolate from a reaction of 15 with methylamine was 20, arising through an intermediate similar to 18 and this was followed by a subsequent reaction of the intermediate with another mole of methylamine.

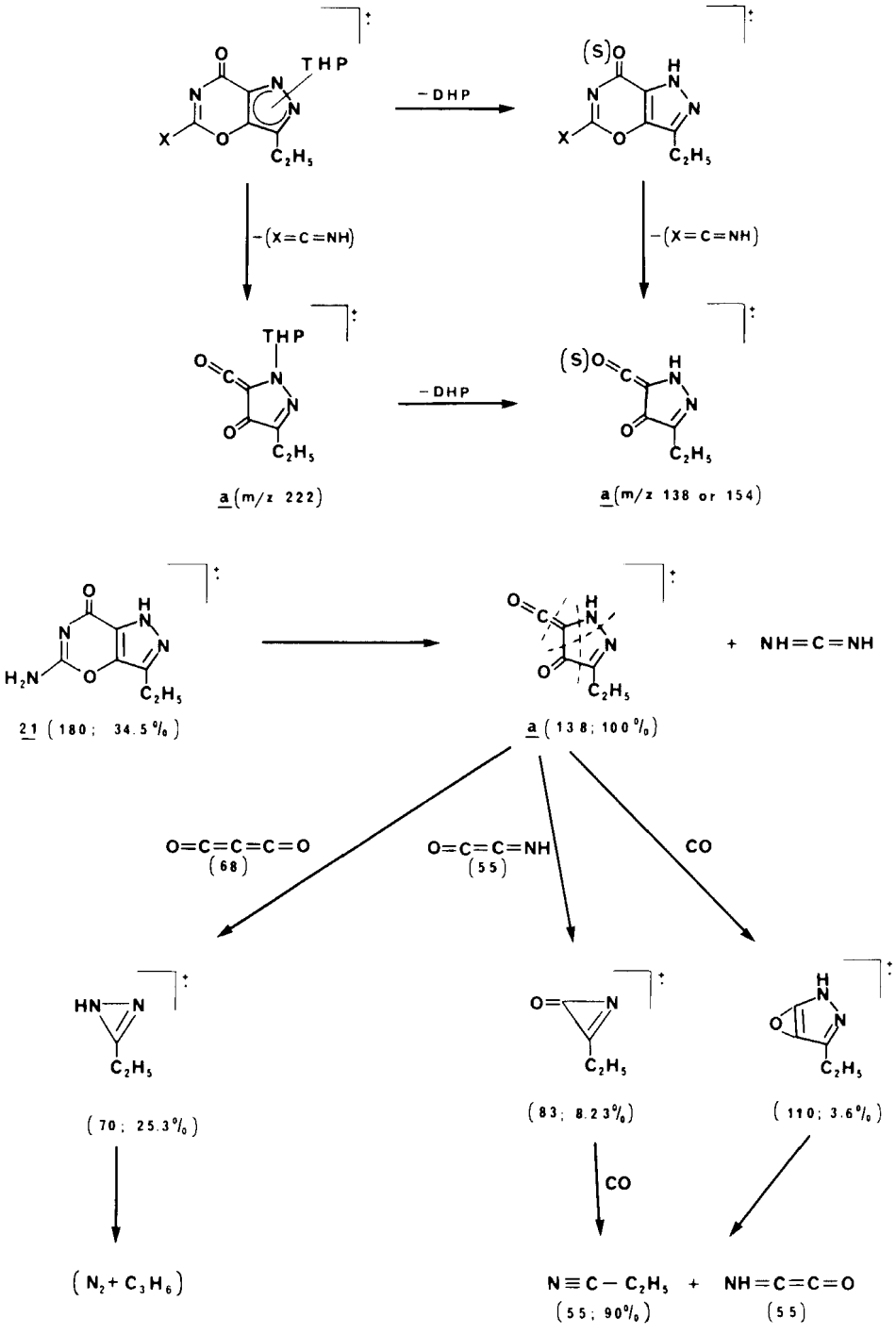
One of the more common procedures used to remove a THP blocking group from a heterocyclic ring nitrogen atom is treatment with a water or methanol solution of HCl. The conditions used were very diverse and varied from a few drops of 0.1 N HCl at room temperature[30] to heating at reflux for 18 hours in a MeOH:conc HCl:H_2O/8:3:1 solution.[40] Our initial investigations involved deblocking 17 with 1 N HCl in a methanol-water solution. However, the reactions were very slow and usually resulted in a complicated mixture of compounds which were difficult to separate. When the HCl concentration was sharply increased, a mixture of two products was obtained. The minor product, more soluble in chloroform, showed a strong singlet at δ 3.7 which was assigned to the CH_3O group of 22a. The major product was purified by HPLC and was also success-

fully deblocked according to ^{1}H NMR spectral data. However, elemental analysis showed that the product contained what appeared to be an additional one mole of water while the ^{1}H NMR spectrum indicated that perhaps what we had in hand was a ring opened product. The mass spectrum of 22 as well as a $FeCl_3$ test confirmed that ring opening of 21a to afford 22 had indeed taken place. We subsequently found that when HCl gas was passed through a cold methylene chloride solution containing 17 for 2-3 minutes, a white precipitate (probably a mixture of 21a and 21) was obtained. Treatment of the solid with DMF containing Et_3N furnished pure 21. A similar procedure was then employed to obtain the deblocked derivatives 3, 12, 13, 14 and 16.

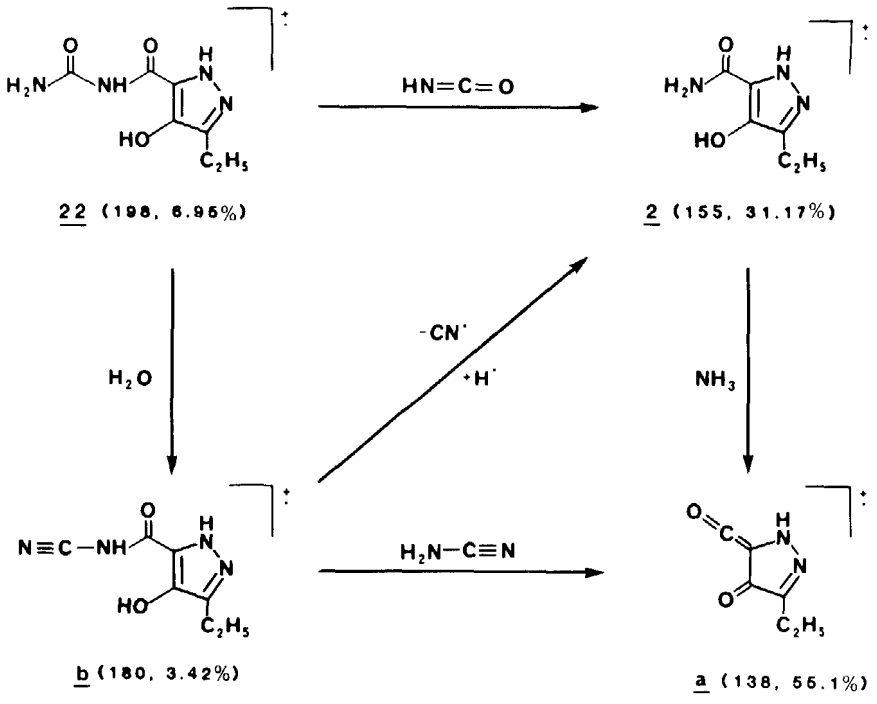
During this study, we found that the mass spectra of these 3,5,7-trisubstituted pyrazolo[3,4-e][1,3]oxazines are very characteristic and similar to other bicyclic 1,3-azines.[41,42] The molecular ions (or M + 1) are detectable but exhibited a variation in intensity from 16 to 58% and were much lower for the THP-protected compounds (1-11%). It appears that a one step primary cleavage of the retro-Diels-Alder type results in the formation of "diene" (X=C=NH) and "dienophile"(a) fragments (Scheme 11). A peak at m/z 138 (or 222 for the THP pro- tected series) is one of the major peaks. According to this frag- mentation pattern, compounds 3, 12 and 13, 14, gave the fragments a m/z 138 and 154, respectively. The peak at m/z 85 was, as a rule, the dominant peak (100%) for the THP-protected compounds.

Assumed pathways of fragmentation for a are shown in Scheme 11. Unlike some of the other compounds, decarbonylation was not involved in the major pathway for the fragmentation of a. A peak at m/z 110 was usually small and not always observed. Peaks at m/z 55, 83 and 70 represent predominant fragments for these compounds.

In contrast to the bicyclic pyrazolo[3,4-e][1,3]oxazines, a molecular ion for the related open-chain derivatives of 2 and 7 was usually lower in intensity and underwent at least a two step frag- mentation to afford the basic fragment a (m/z 138). All of the analyzed O- and N- substituted derivatives of 2 and 7 showed a peak



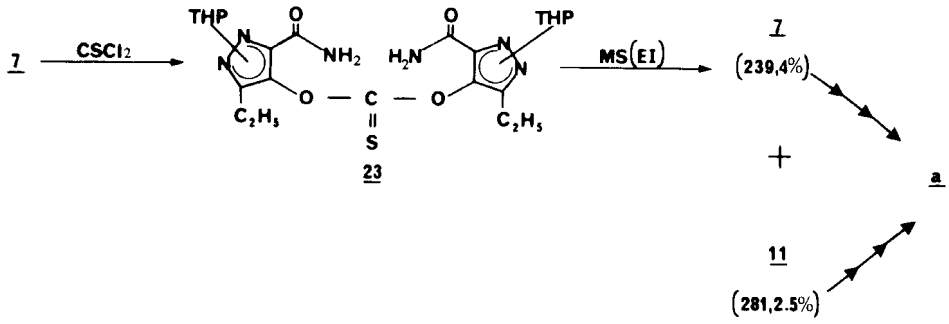
SCHEME 11



SCHEME 12

at m/z 155(239) which corresponds to the carboxamide 2 which then underwent a regular fragmentation. The relative intensity of the peak at m/z 155 (239) was variable. In some cases this intensity was less than 5% but sometimes would be as high as 48%. Therefore, the presence of the peak at m/z 155 (239) is very characteristic in the mass spectrum of the open-chain compounds and may be useful for a structure determination when the molecular ion is too small to be reliable and the fragment b gives the highest peak in the spectrum.

An excellent example of this is as follows: 7 with thiophosgene provided a product (23) which was isolated and showed a negative $FeCl_3$ test and MS (EI) with the largest mass peak at m/z 281. This peak at m/z 281 is equal to the molecular ion peak of the bicyclic compound 11, but the MS of 23 also revealed peaks at m/z 239 (7) and 155 (2) which, as it was mentioned above, could not be formed from 11. This suggested an open-chain structure for 23 and the possibility of the structure illustrated in Scheme 13 in spite of the absence of a molecular ion peak at m/z 520. It would appear

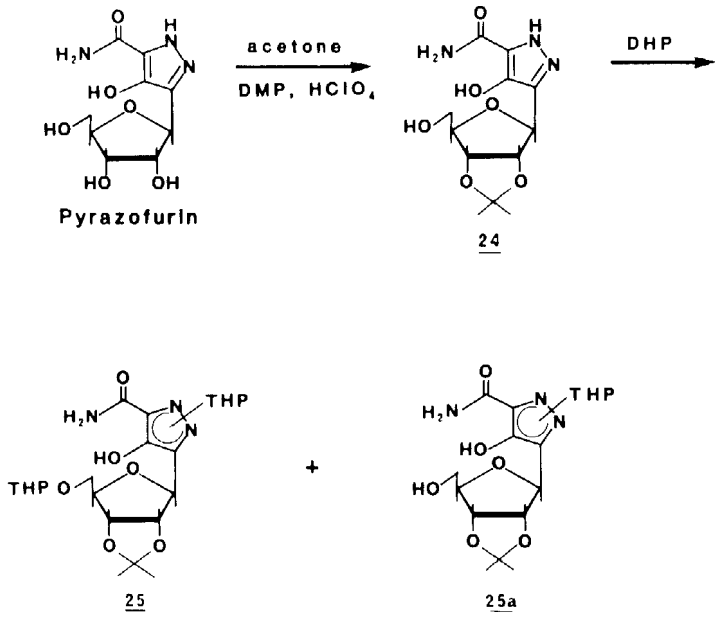


SCHEME 13

that 7 functions as a leaving group under electron impact conditions to furnish compound 11. Compounds 7 and 11 then followed the fragmentation pathways described above to afford fragment a (Scheme 13). The ^{1}H NMR spectrum (broad doublet of NH_2 at 7.2 ppm), IR data and elemental analysis provided additional support for the structural assignment of 23.

Our preliminary studies on the model compounds, vide supra, allowed us to apply these synthetic approaches to the nucleoside series using pyrazofurin as our starting material. The nucleoside synthesis can be separated into four major steps: 1) synthesis of pyrazofurin protected at the 1 (2), 2',3',5' positions; 2) ring closure by 1,1'-carbonyldiimidazole to a 5,7-dioxoderivative ring closure by 1,1'-thiocarbonyldiimadazole to afford 5-thione-7-oxo derivatives; 3) transformation of the 5-thione group into a methylthio and/or an amino groups; 4) removal of blocking group to obtain target compounds.

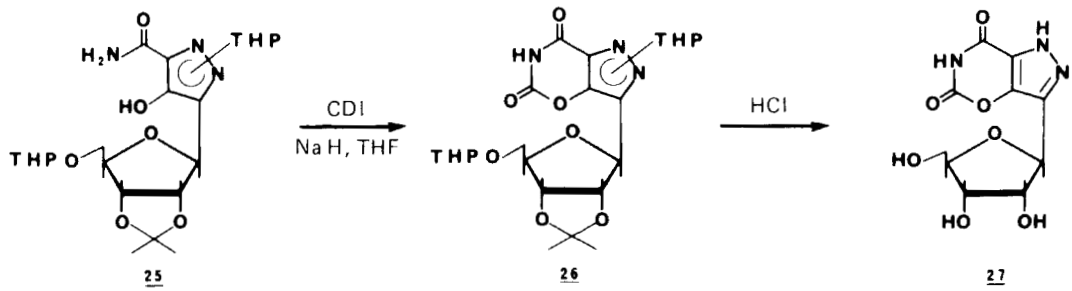
The protected derivative (25) of pyrazofurin was prepared by a two step synthesis. The 2',3'-O-isopropylidene derivative 24 had been obtained and characterized previously as an intermediate in a total synthesis of pyrazofurin[43]. Compound 24 was also prepared from pyrazofurin using p-toluene sulfonic acid as a catalyst[26]. The latter method was not described in detail and appeared to be unsuitable for our preparative synthesis because it required three days, PTLC for the separation, and no yield or analytical data were reported. Therefore, compound 24 was synthesized in our laboratory by a modified method[44]. 2,2-Dimethoxypropane with $HClO_4$ as a



SCHEME 14

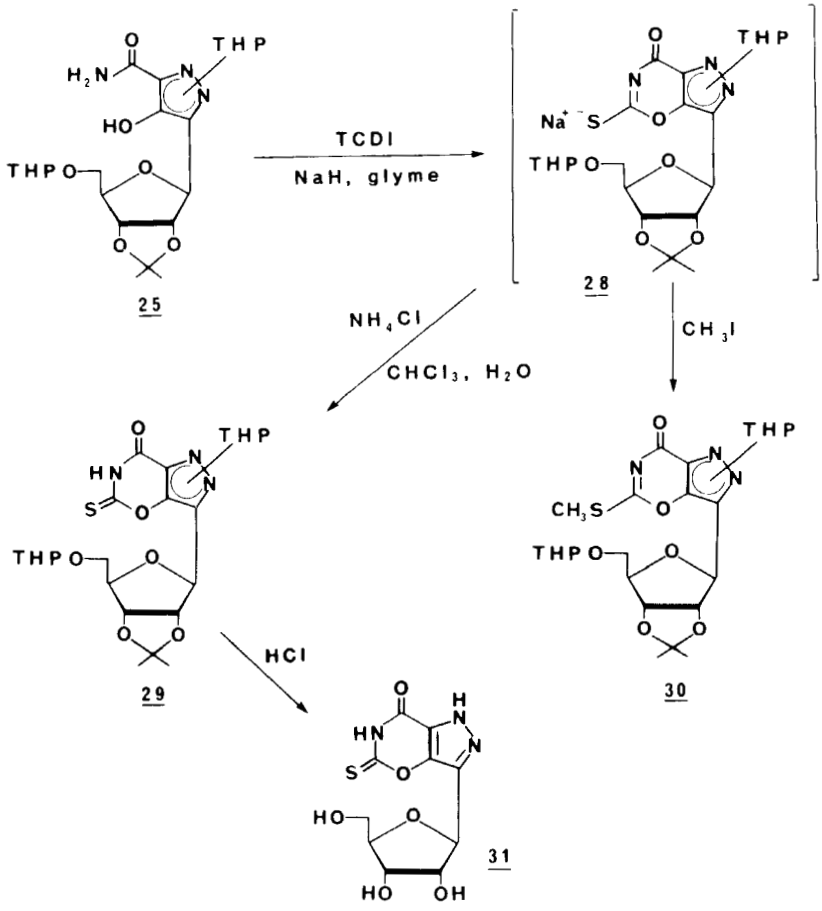
catalyst furnished a complete transformation of pyrazofurin into 24 under mild conditions (yield 80 - 85%). The 1,5'-di-tetrahydropyranyl derivative 25 was obtained under conditions similar to those we had previously developed for the protection of the model compound, 2 to 7 but in lower yield (up to 50% instead of 65%). This lower yield was most likely due to the formation of two side products which were observed by TLC. One of the side products had a lower TLC mobility and, was found to be the mono THP derivative 25a. This side product was usually obtained when the ratio of 24 to DHP was 1 to 4, but when this ratio was changed to 1:8 a new product with a much higher TLC mobility was formed as the sole reaction product. This product showed a positive $FeCl_3$ test and it was subsequently established that there were three THP groups present. The mono THP product 25a was isolated during the purification of 25 by column chromatography and the structure of 25a was confirmed by elemental analysis and MS (CI, CH_4).

In contrast to the model compound 7, the ring closure of 25 with CDI did not occur when heated in THF at reflux. When sodium hydride was used, the nucleoside 26 was obtained in about 80% yield.



SCHEME 15

When we used the procedure (90% CF_3COOH) reported[43] for the deblocking of 24 in an attempt to deblock 26, we obtained a complicated mixture which was difficult to separate. The protected compound 26 was successfully deblocked using our general procedure



SCHEME 16

of passing HCl gas through a solution of the compound in dichloromethane at 0-3° for 5 min. A precipitate was formed in 1-2 min but the reaction mixture was kept at 0° for an additional 2 hrs to insure that complete deblocking had occured. The nucleoside 27 obtained from this reaction, in 75% yield, showed one major spot by TLC and one major peak when it was analyzed by HPLC (MCH-5, 15cm, RP-18, 2% MeOH in H_2O). The sample was finally purified by LPLC (Lobar "B", RP-8, H_2O) and gave analytically pure 27 in ~ 57% yield. UV and IR spectral data were essentially the same as those observed for the model compound 3. The ^1H NMR (D_2O, 360 MHz) spectrum showed peaks at δ 4.90 (d, 1H, H1', $J_{1'-2'}$ = 6.8Hz), 4.30 (ps t, 1H, H2'), 4.09 (t, 1H, H3'), 3.95 (q, 1H, H4'), 3.63 (m, 2H, H5',H5"). The MS spectrum (CI, CH_4) revealed a peak at m/z 286 $(M + 1)^+$. These data confirmed that ring opening had not occurred and that the structure assigned to 27 is indeed 3-(β-D-ribofuranosyl)-pyrazolo[3,4-e][1,3]oxazin-5,7-dione.

Ring closure of 25 by TCDI was performed in dry glyme with NaH as a catalyst to afford the intermediate 26a. This intermediate was converted into compound 29 which was pure by TLC. The UV and IR spectral data for this compound were similar to those obtained for the model compound. Elemental analysis for this compound were also correct. Deblocking of 29 by our standard procedure, gave a slightly yellow solid which according to TLC contained a small amount of impurities. HPLC analysis (Partisil PXS, 10x 2.5 cm, ODS-3, 2% MeOH in H_2O) showed that the major component was about 89% of the mixture and this sample (~ 30 mg) was purified by LPLC (RP-18, 2% MeOH in H_2O). Lyophylization furnished about 15 mg of 31 as a bright yellow powder which provided correct IR spectral data and elemental analysis. The UV spectrum was essentially identical to the spectrum obtained for the model compound (Fig. 1).

The 5-methylthio derivative 30 was obtained under conditions similar to those we had developed for the model compound. Direct alkylation of the intermediate 28 and purification by column chroma-

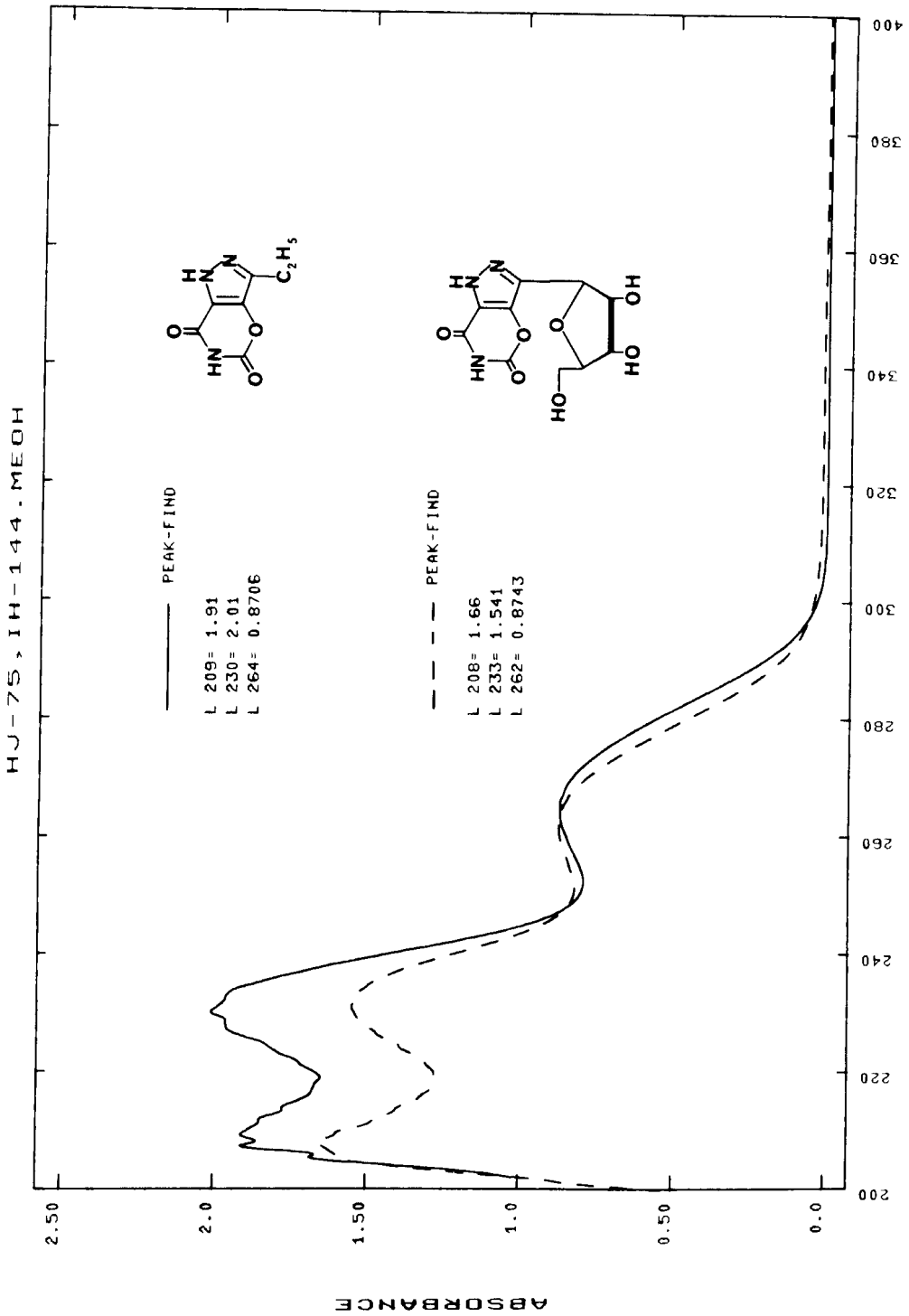
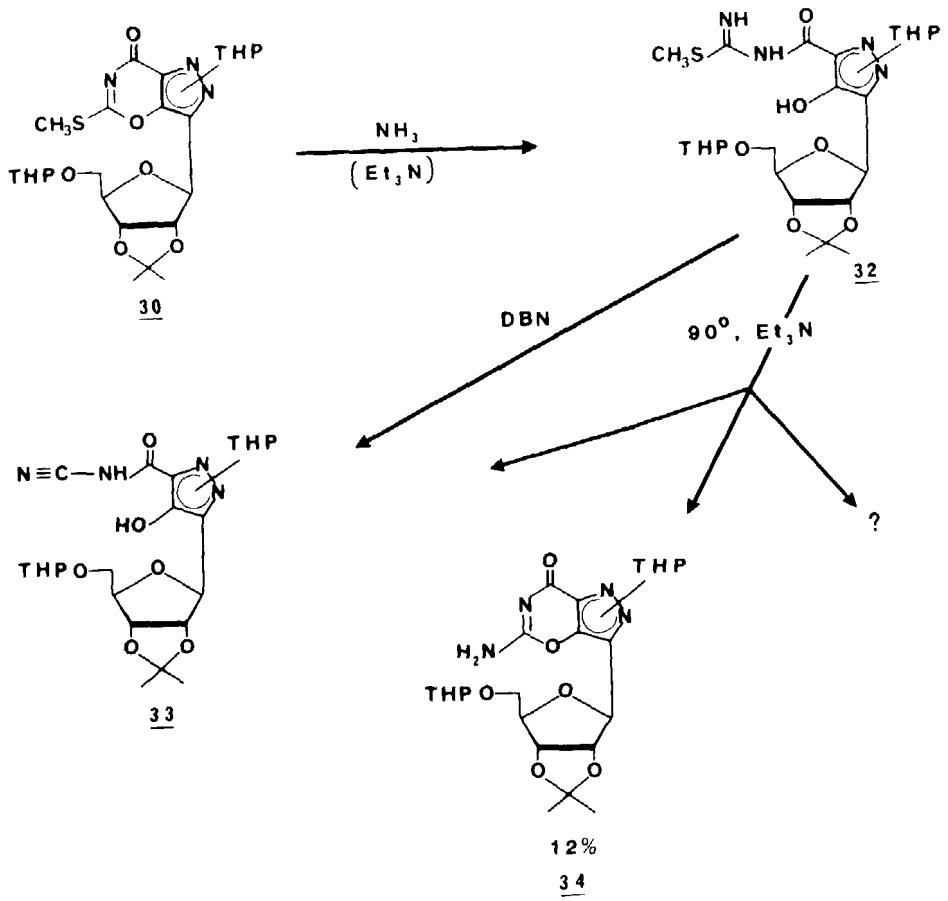


FIG. 1



SCHEME 17

tography yielded 47.6% of 30 which showed correct spectral and elemental analysis.

The amination of 30 by ammonia, unlike the model synthesis, gave only one compound (32). Several different reaction conditions were then investigated in an effort to effect a ring closure of 32. The reaction was very slow and was accompanied by at least two side reactions. One of the side reactions furnished compound 33 and it was the major product when a strong base (DBN) was used as a catalyst. The presence of a CN-group was confirmed by IR spectral data. Another side reaction leading to decomposition (no UV absorption higher then 210 nm was observed) became a major reaction

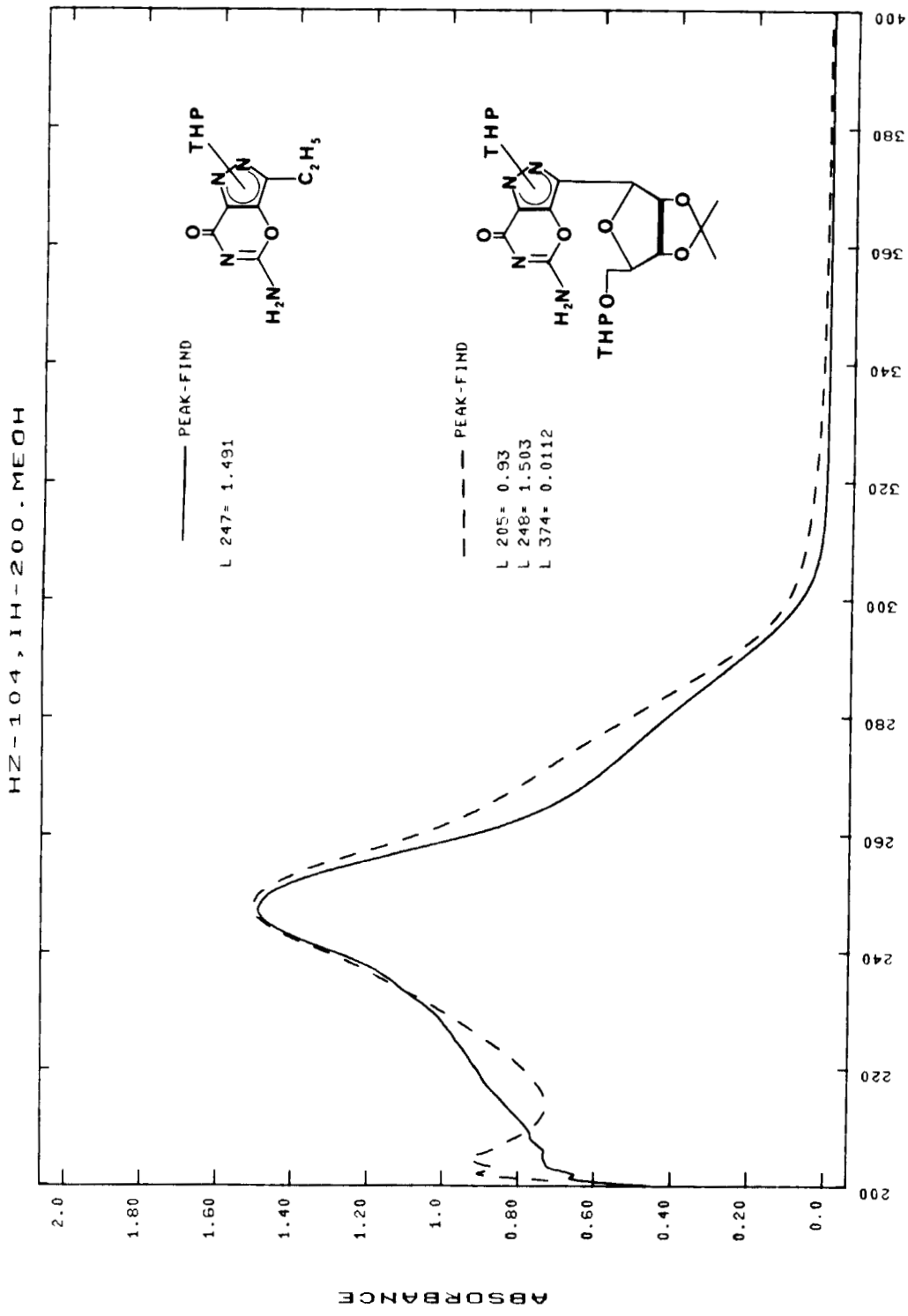
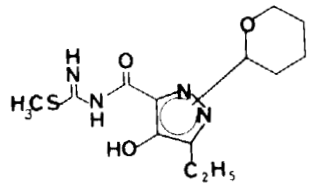
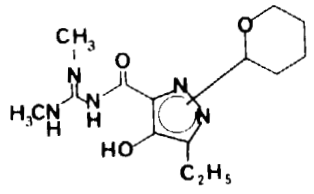
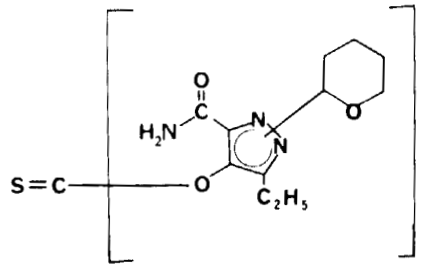


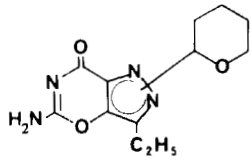
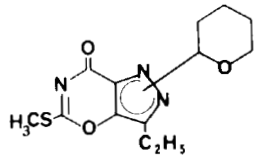
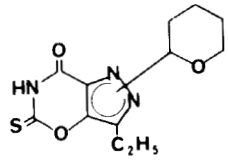
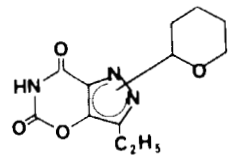
FIG. 2

Table 1. L1210 *in Vitro* Cytotoxicity Testing of Precursors to Pyrazolo[3,4-*e*][1,3]oxazines

Compound No.	Structure	Growth rate at 10^{-4}M (% of control)	ID_{50} (M)
18		97	--
20		111	--
23		82	$>10^{-4}$M

when the reaction time and/or temperature were increased. This study allowed us to obtain the amino derivative 34 in ~ 15% yield by varying the reaction conditions. The best conditions, so far, were triethylamine in toluene at 90° and careful monitoring of the

Table 2. L1210 *in Vitro* Cytotoxicity Testing of Tetrahydropyranyl Pyrazolo[3,4-*e*][1,3]oxazines

Compound No.	Structure	Growth Rate at 10^{-4}M (% of control)
17		100
15		89
11		100
9		100

reaction by TLC to determine the optimum time for terminating the reaction. Compound 34 had to be carefully purified to eliminate a small amount of 32. Compound 34 also showed spectral data (Fig. 2)

Table 3. L1210 *in Vitro* Cytotoxicity Testing of Pyrazolo[3,4-e][1,3]oxazines

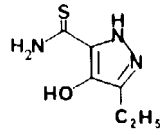
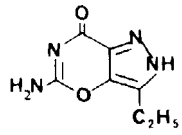
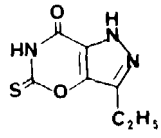
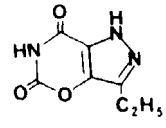
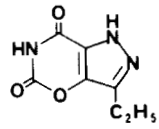
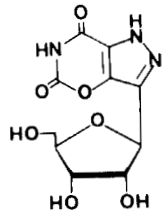
Compound No.	Structure	Growth Rate at 10^{-4} M (% of control)
8		100
21		100
13		100
12		100
3		100

Table 4. L1210 in Vitro Cytotoxicity Testing of Pyrazolo-[3,4-e][1,3]oxazine Nucleoside

Compound No.	Structure	Growth Rate at 10^{-4}M (% of control)	ID_{50} (M)
3		100	---
27		0	2.7×10^{-7}

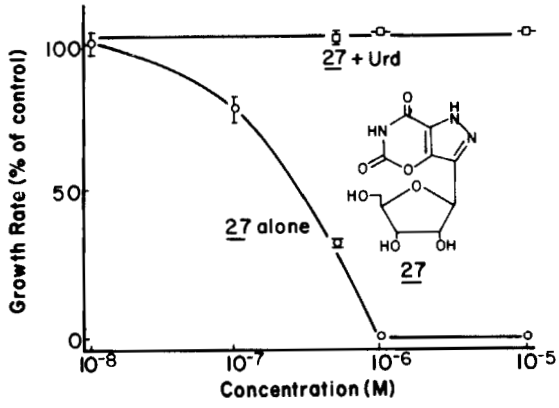


Figure 3. L1210 Cell Growth Inhibition by the pyrazolo[3,4-e][1,3] oxazine anolog of xanthosine (27). Growth rate (the slope of the semilogarithmic plot of cell number against time, as a percentage of the control rate) is plotted against the concentration of 27, for 27 alone (-O-) and for 27 combined with 1 or 2 mM Urd (-□-). Bars = range; n=2.

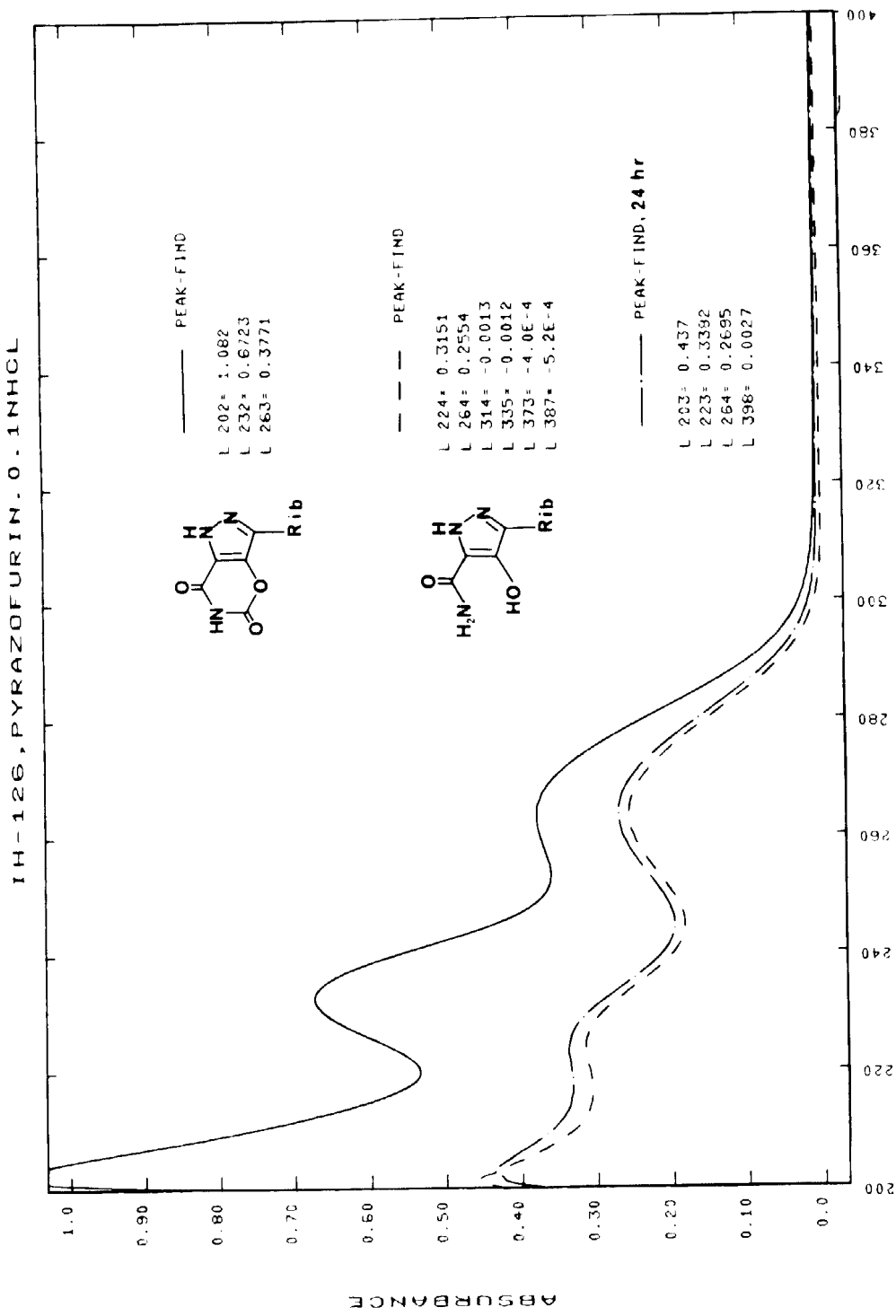


FIG. 4

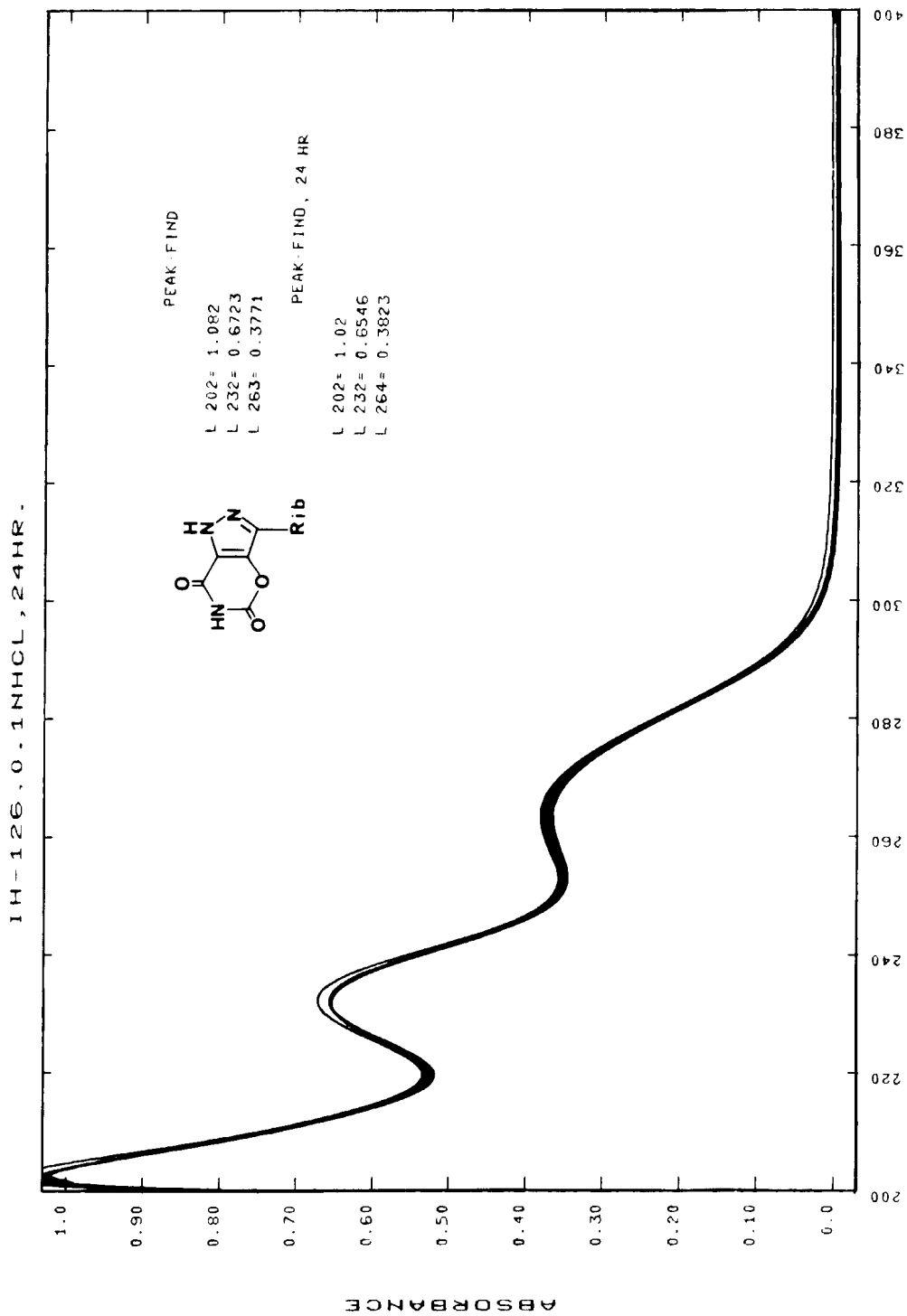


FIG. 4

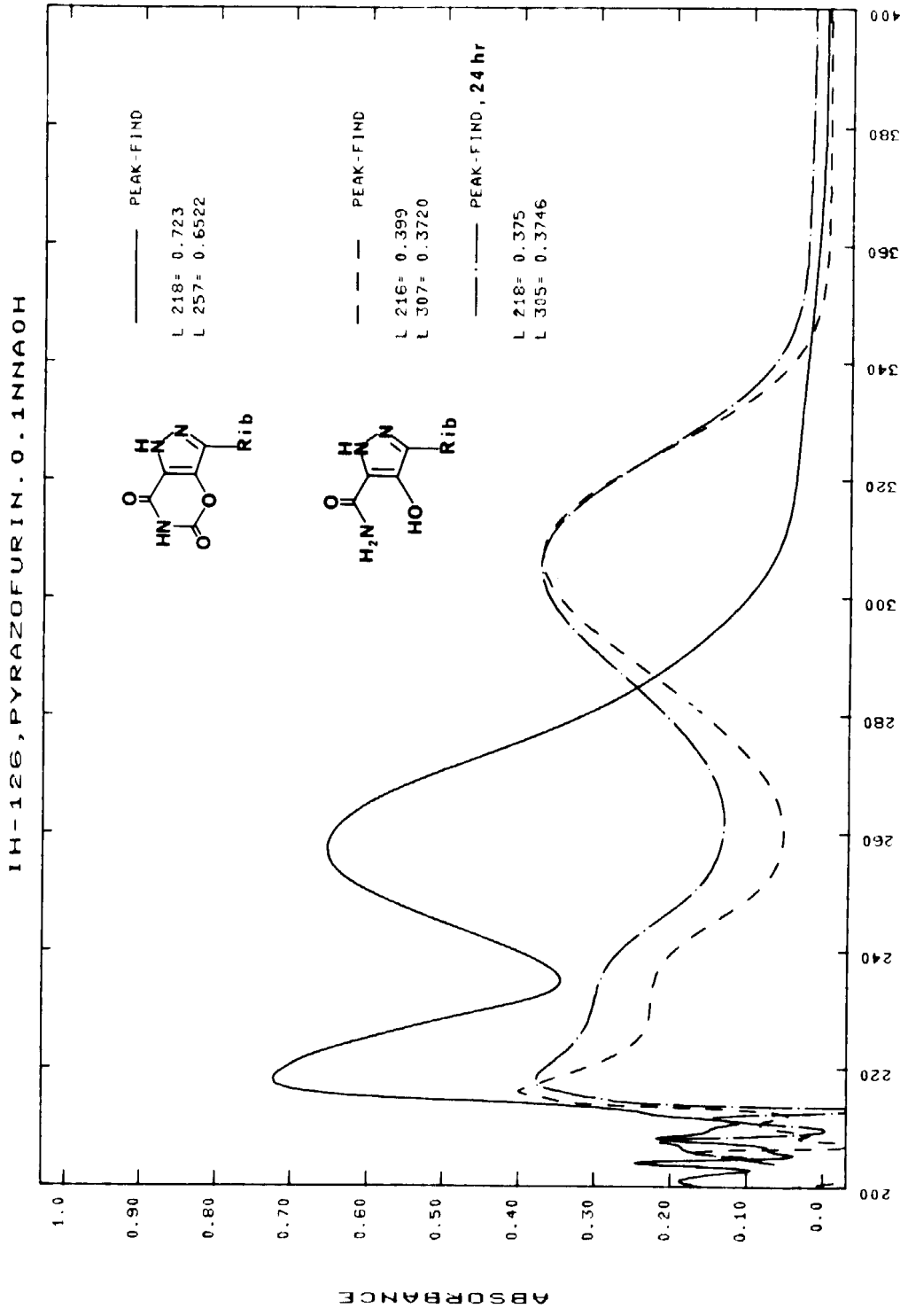


FIG. 5

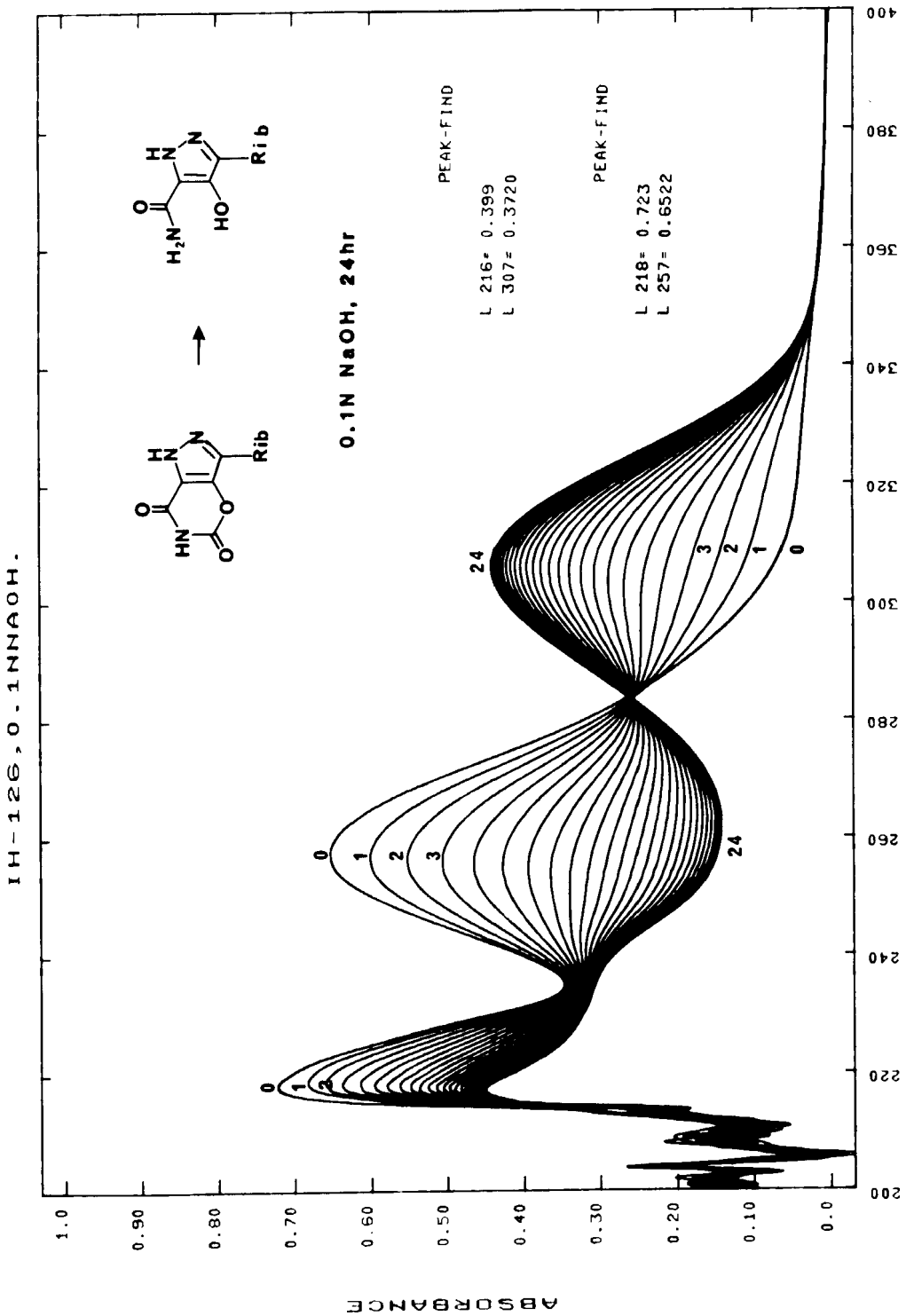


FIG. 5

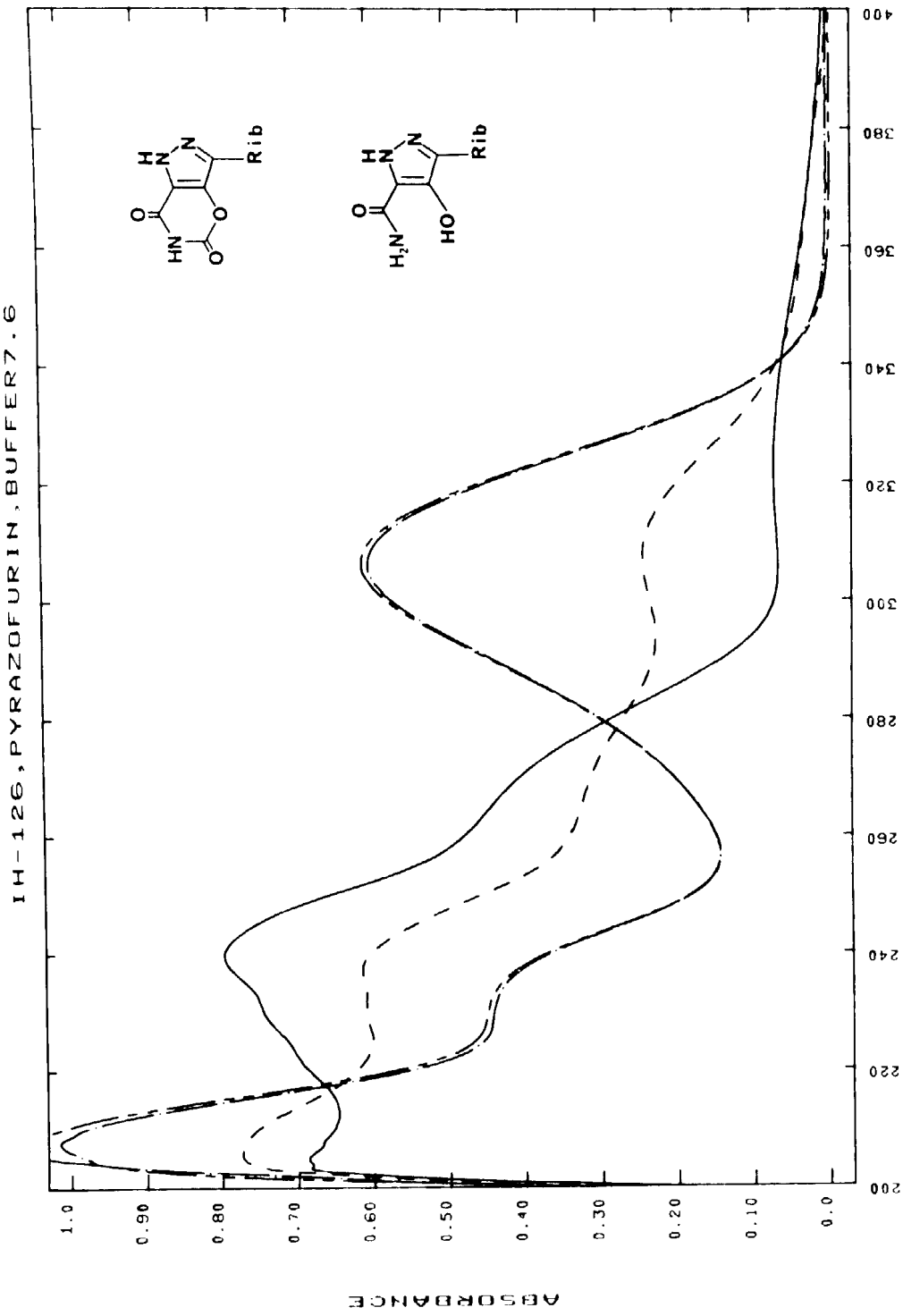


FIG. 6

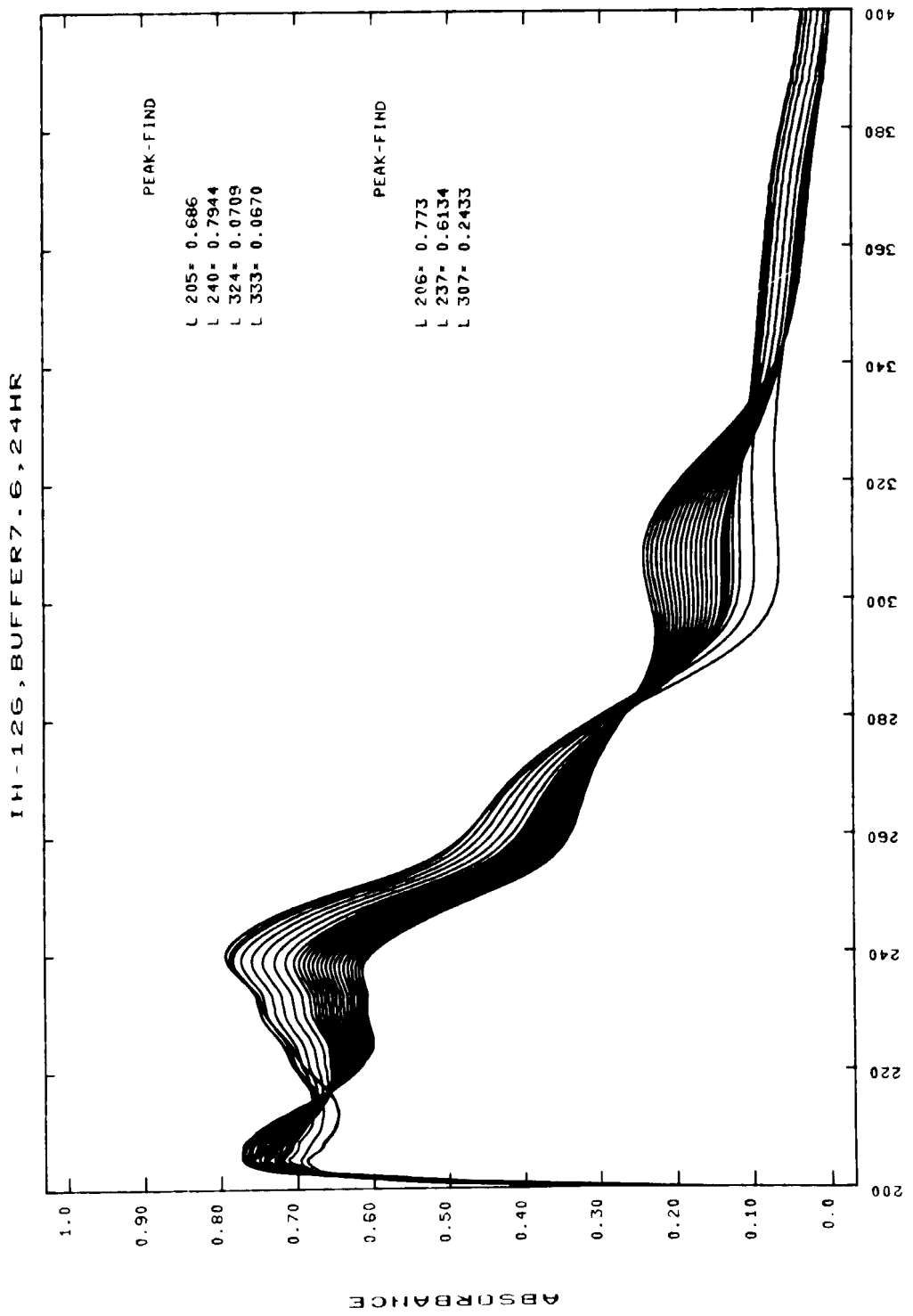


FIG. 6

very similar to the model compound. A sample of 34 was 95% pure according to HPLC analysis. We should now be able to remove the blocking groups using the procedure developed in our laboratory and this will provide the guanosine analog.

The potential of these compounds as antineoplastic drugs was investigated using L1210 cells in vitro. The pyrazole precursors to the pyrazolo[3,4-e][1,3]oxazines (Table 1), the tetrahydropyranyl ethyl pyrazolo[3,4-e][1,3] oxazines (Table 2), and the ethyl pyrazolo[3,4-e][1,3]oxazines (Table 3) did not significantly inhibit growth of the cells. In contrast, the ribosyl derivative (27) of the xanthine analog (3) strongly inhibited the growth of L1210 cells with an $ID_{50} = 2 \times 10^{-7}$ (Table 4) (ID_{50} = concentration required to decrease the growth rate of the cells to 50% of the control rate). This growth inhibition could be totally prevented by the simultaneous addition of uridine to the cell cultures (Figure 3), suggesting that 27 might exert its growth-inhibitory effect by inhibiting pyrimidine de novo synthesis. A possible mechanism for such an effect might be cleavage of the oxazine ring of 27 to provide pyrazofurin, which is known to block pyrimidine de novo synthesis by inhibiting orotidylate decarboxylase[45]. Therefore, the stability of the pyrazolo[3,4-e][1,3]oxazine ring of 27 under various conditions was investigated.

Compound 27 was found to be stable in acid, but unstable in base. Specifically, in 0.1 N HCl, the UV spectrum of 27, as well as the UV spectrum of pyrazofurin, remained unchanged up to 24 hr, the longest time interval tested (Figure 4). In 0.1 N NaOH, on the other hand, 27 was essentially converted to pyrazofurin within 24 hr, as shown by the changes in the UV spectrum (Figure 5). Under conditions approximating those in the L1210 cell cultures, namely in phosphate buffered saline (0.025 M KH_2PO_4, 0.1 M NaCl, pH 7.6), 27 was converted more slowly to pyrazofurin (Figure 6), with approximately one third of 27 being converted into pyrazofurin in 24

hr at room temperature. Thus, it appears possible that the cytotoxicity of 27 can be accounted for by its conversion to pyrazofurin in the L1210 cell cultures. Further studies are in progress to test this hypothesis directly.

Acknowledgement:

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