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# Cryptopleurine—An Inhibitor of Translocation†

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ABSTRACT: Ribosomes from a cryptopleurine-resistant mutant of yeast were analyzed to determine the subunit localization of the resistance alteration. As was previously reported (Grant, P., Sanchez, L., and Jiminez, A. (1974), *J. Bacteriol. 120,* 1308), in vitro resistance of polyphenylalanine synthesis to cryptopleurine was conferred by 40S subunits from the mutant. Binding studies with sensitive ribosomes were carried out in order to identify the subunit binding site for cryptopleurine. Over the range of concentrations which inhibited polyphenylalanine synthesis, binding was proportional to concentration, so that a unique binding site could not be detected. Furthermore, binding to isolated subunits was about fourfold greater than to 80S ribosomes, suggesting that nonspecific binding was sensitive to the condition of the particles. Model systems were developed in order to determine which step of the elongation cycle was inhibited by cryptopleurine. Elongation factor 1 dependent binding of Phe-tRNA to ribosomes was not inhibited by cryptopleurine concentrations, which inhibited polyphenylalanine synthesis. The initial rate of *N*-acetylphenylalanylpuromycin formation was inhibited when $10^{-5}$ M cryptopleurine was added prior to translocation, but not when added after. Little inhibition was observed in either case when mutant ribosomes were used. These results suggest that cryptopleurine primarily inhibited translocation.

A variety of studies on inhibition of protein synthesis by cryptopleurine have suggested a primary effect on elongation. Stabilization of polysomes was observed in cell extracts after exposure to the inhibitor, suggesting that elongation, or termination, was blocked. Concentrations similar to those which blocked cell growth inhibited poly(U)[1]-directed polyphenylalanine synthesis or elongation of endogenous nascent chains, implying that some step in elongation was affected (Donaldson et al., 1968; Haslam et al., 1968; Battaner and Vazquez, 1971; Skogerson et al., 1973; Grant et al., 1974).

Inhibition of peptidyl-puromycin formation suggested that peptidyl transfer was the affected reaction (Pestka et al., 1972). Others reported no inhibition of peptidyl transfer and suggested that cryptopleurine inhibited translocation (Huang and Grollman, 1972; Battaner and Vazquez, 1971; Barbacid et al., 1975). Data were also presented that 60S ribosomal subunits were preferentially inactivated by the inhibitor (Battaner and Vazquez, 1971).

In spite of the evidence that cryptopleurine interfered with a 60S ribosomal function, in vitro resistance to the inhibitor was due to altered 40S subunits from resistant mutants (Grant et al., 1974). If cryptopleurine specifically affects a 60S ribosomal function, 40S resistance suggests a novel and interesting interaction between the two ribosomal subunits. Either inhibition or resistance would necessarily be accomplished through subunit–subunit interaction.

The present study was undertaken in order to characterize further the ribosomal alteration resulting from mutation of the *cry 1* gene and to establish more definitively the stage of protein synthesis affected by cryptopleurine.

## Materials and Methods

Biochemicals and reagents were obtained from standard sources as previously described (Skogerson et al., 1973; Skogerson and Wakatama, 1976).

*Polyphenylalanine Synthesis.* Ribosomes, elongation factors, and [$^3$H]Phe-tRNA (sp act. 7.79 Ci/mmol) were prepared as previously described (Skogerson et al., 1973; Skogerson and Wakatama, 1976). Ribosomal subunits were prepared by the high-salt dissociation method of Martin and Wool (1968). Standard reaction mixtures of 0.05 ml contained 60 mM Tris–acetate, pH 7.0, 50 mM $NH_4Cl$, 8 $\mu$g of poly(U), 12 mM $MgCl_2$, 1.0 mM GTP, 20 mM dithiothreitol, 6 $\mu$g of EF-1, 0.04 $\mu$g of EF-2, 0.1 $\mu$g of EF-3, 0.04 $A_{260}$ unit of 80S or 0.04 $A_{260}$ unit of 40S and 0.06 $A_{260}$ unit of 60S subunits, and 10–15 pmol of [$^3$H]Phe-tRNA. Reaction times of 4 or 5 min were used in order to obtain initial rates. Hot $CCl_3CO_2H$

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[1] Abbreviations used are: EF, elongation factor, EF-3, yeast ribosome-dependent GTPase described previously (Skogerson and Wakatama, 1976); *N*-AcPhePuro, *N*-acetylphenylalanylpuromycin; DEAE, diethylaminoethyl; GTP, guanosine triphosphate; GTPase, guanosine triphosphatase; poly(U), poly(uridylic acid).

insoluble radioactivity was determined as previously described (Skogerson et al., 1973).

Binding of [$^3$H]Phe-tRNA was carried out in reaction mixtures identical to those used for polyphenylalanine synthesis, except that 0.6 $A_{260}$ unit of ribosomes was used and EF-1 was the only exogenous factor added. Reaction mixtures were incubated at 4 °C and the ribosome-bound [$^3$H]Phe-tRNA was determined as described (Skogerson and Wakatama, 1976).

*N-Acetylphenylalanylpuromycin Synthesis.* Translocation-dependent formation of *N*-Ac[$^3$H]PhePuro was measured by a modification of the procedure used by Siler and Moldave (1969). *N*-Ac[$^3$H]Phe-tRNA was prepared by acetylation of [$^3$H]Phe-tRNA according to Haenni and Chapeville (1966) and contained no detectable unacetylated [$^3$H]Phe. Optimal activity of this reaction required the addition of EF-2, EF-3, and another protein, factor P. Factor P was obtained by DEAE-Sephadex chromatography similar to that described for elongation factors (Somasundaran and Skogerson, 1976) and had no detectable effect on polyphenylalanine synthesis.

Binding of *N*-Ac[$^3$H]Phe-tRNA to ribosomes was carried out in 0.04 ml of reaction mixtures containing 60 mM Tris-acetate, pH 7.0, 50 mM $NH_4Cl$, 24 mM $MgCl_2$, 0.1 $A_{260}$ unit of ribosomes, 8 μg of poly(U), and 12 pmol of *N*-Ac[$^3$H]-Phe-tRNA. Translocation was accomplished in 0.11 ml of reaction mixtures containing 60 mM Tris-acetate, pH 7.0, 50 mM $NH_4Cl$, 9 mM $MgCl_2$ (final concentration), 30 mM dithiothreitol, 4 ng of EF-2, 0.02 μg of EF-3, 0.1 μg of factor P, 0.7 mM GTP, and 0.04 ml of the binding reaction mixture. Under these conditions, the rate of translocation was limited by the concentration of EF-2. After 20 min at 30 °C, puromycin was added to give a final concentration of 1 mM. Formation of *N*-Ac[$^3$H]PhePuro was stopped at various times by dilution with 1 ml of 50 mM Tris-acetate, pH 7.0, and the product extracted into 1.5 ml of ethyl acetate (Leder and Bursztyn, 1966). One-milliliter samples of the ethyl acetate phase were counted with 3 ml of Aquasol (New England Nuclear) at an efficiency of 18% and the results were corrected for 1.5 ml.

*Cryptopleurine Binding.* Reaction mixtures of 1 ml contained 60 mM Tris-acetate, pH 7.0, 50 mM $NH_4Cl$, 12 mM $MgCl_2$, cryptopleurine concentrations ranging from $10^{-6}$ to $10^{-5}$ M, and either 5 $A_{260}$ units of ribosomes, 1.7 $A_{260}$ units 40S subunits, or 3.4 $A_{260}$ units of 60S subunits. After 5 min at 30 °C, the reaction mixtures were centrifuged at 105 000*g* for 30 min at 0 °C. Unbound cryptopleurine in the supernatant was determined by fluorescence using a Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A. Emission at 377 nm was measured with excitation at 320 nm. Ribosomal supernatants did not affect the fluorescence of cryptopleurine under the conditions tested. Standard curves were prepared relating intensity of fluoresence to cryptopleurine concentration in ribosomal supernatant. Absolute concentrations of cryptopleurine were determined by the extinction at 258 nm (Bradsher and Berger, 1958).

## Results

*Subunit Studies.* We previously reported that mutations mapping 2.1 centimorgans from the mating locus on chromosome III of *S. cerevisiae* resulted in ribosomes resistant to inhibition by cryptopleurine (Skogerson et al., 1973). While determining which subunit was altered by the mutation to resistance, we found that 40S subunits from cryptopleurine-resistant mutants were less active than those from the parent in poly(U)-directed polyphenylalanine synthesis. Shown in Table I are the relative activities of mutant or parental 40S subunits in the presence of a fixed excess of either mutant or parental 60S particles. Polyphenylalanine synthesis was 30 to 50% less when the 40S subunits were from the mutant, compared to the parent, regardless of the source of the 60S particles. Similar observations were reported by Grant et al. (1974).

TABLE I: Relative Activities of Parental and Mutant Ribosomes and Ribosomal Subunits.

| Ribosomes | Sp Act.[a] PHE per Particle per Min |
|---|---|
| Parental 40S, parental 60S[b] | 1.74 |
| Parental 40S, mutant 60S | 1.67 |
| Mutant 40S, parental 60S | 0.90 |
| Mutant 40S, mutant 60S | 1.15 |
| Parental 80S[c] | 4.46 |
| Mutant 80S | 4.30 |

[a] A specific activity of 1 phenylalanine polymerized per min per particle corresponds to 53 pmol per $A_{260}$ 40S or 18 pmol per $A_{260}$ 80S per min. [b] Rates of polyphenylalanine synthesis were measured as a function of 40S concentration in the presence of 0.03 $A_{260}$ 60S subunits. Specific activities were determined from the linear portion of each curve. [c] Rates of polyphenylalanine synthesis were measured as a function of 80S concentration.

TABLE II: Ribosomal Subunit Localization of in Vitro Resistance to Cryptopleurine.

| Ribosomes | pmol of Phe polymerized[a] Control | $10^{-6}$ M Cryptopleurine | % Inhibition |
|---|---|---|---|
| Parental 40S, parental 60S | 1.87 | 0.90 | 52 |
| Parental 40S, mutant 60S | 1.99 | 0.90 | 55 |
| Mutant 40S, parental 60S | 1.34 | 1.11 | 17 |
| Mutant 40S, mutant 60S | 1.20 | 0.94 | 22 |
| Parental 80S | 3.60 | 1.44 | 60 |
| Mutant 80S | 3.47 | 2.53 | 27 |

[a] Polyphenylalanine synthesis was carried out as described in the text in the presence and absence of $10^{-6}$ M cryptopleurine.

The activities of the ribosomes from which the subunits were derived were similar, suggesting that 40S particles from the mutant were less stable to the separation procedure than those from the parent. Loss of 40S activity was observed in subunit preparations from two independently isolated mutants, suggesting a possible change in 40S particles as a result of the mutation to cryptopleurine resistance. When the sensitivity of polyphenylalanine synthesis to cryptopleurine was determined in systems using subunits from either parent or mutant, the in vitro resistance of mutant ribosomes was found to be exclusively a property of the 40S subunits (Table II). We conclude that mutation of the gene, *cry 1*, results in an alteration of the 40S subunit, which confers resistance of the ribosome to cryptopleurine and secondarily renders the 40S subunits less stable to isolation and purification.

*Binding Studies.* Battaner and Vazquez (1971) reported that cryptopleurine specifically inactivated 60S subunits and inhibited either peptidyl transfer or translocation. If crypto-

TABLE III: Cryptopleurine Binding to Parental or Mutant Ribosomes and Ribosomal Subunits.

| Ribosomes | mol of Cryptopleurine Bound per mol Particle[a] |
|---|---|
| Parental 80S | 9 |
| Mutant 80S | 5 |
| Parental 40S | 16 |
| Parental 60S | 35 |
| Mutant 40S | 22 |
| Mutant 60S | 29 |

[a] Binding studies were carried out as described in the text at a cryptopleurine concentration of $5 \times 10^{-6}$ M.

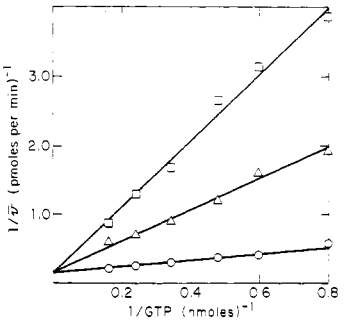


FIGURE 1: Competitive inhibition of polyphenylalanine synthesis by cryptopleurine vs. GTP. Initial rates of polyphenylalanine synthesis were measured at the indicated GTP concentrations in the absence (○) or presence of $10^{-6}$ M cryptopleurine (△) or $2 \times 10^{-6}$ M cryptopleurine (□).

pleurine binds to 60S subunits, the 40S alteration must either prevent binding or overcome the inhibitory effect. If cryptopleurine binds to 40S subunits then inhibition might involve an effect of 40S bound compound on a 60S reaction. In order to distinguish between these possibilities, we attempted to determine the site of binding under conditions similar to those used for polyphenylalanine synthesis.

The method we used to measure binding was a modification of that described by Barbacid and Vazquez (1974) to measure binding of anisomycin to ribosomes. Ribosomes were centrifuged from solutions containing various concentrations of cryptopleurine and the unbound inhibitor concentration was determined in the supernatant by fluorescence. Over the range of concentrations which permitted accurate determination of the bound fraction, the binding was proportional to the concentration. Under these conditions, no specific binding site could be determined. The data obtained at $5 \times 10^{-6}$ M cryptopleurine with mutant and parental ribosomes and subunits are shown in Table III. This concentration inhibited polyphenylalanine synthesis with parental ribosomes about 75%. The binding observed with isolated subunits was much greater than that observed with the 80S preparations, suggesting that the degree of binding was very sensitive to the conditions of the particles. For this reason, we cannot say whether the difference in binding to mutant and parental ribosomes may reflect a structural rather than mutational alteration.

*Mechanism of Inhibition.* In the absence of a direct identification of the binding site for an inhibitor, the mechanism of inhibition is an indirect way to assess how the inhibitor might interact with ribosomes. Employing such an approach, we have attempted to determine which steps in polyphenylalanine synthesis are affected by cryptopleurine.

Analysis of the differential sensitivity of parental and mutant ribosomes was complicated by variability in the sensitivity of the parental system. In an attempt to find the origin of the variability, we measured inhibition as a function of concentration of the various components required for polyphenylalanine synthesis. No effect on inhibition was observed when the concentrations of Phe-tRNA, poly(U), or ribosomes were varied. However, the degree of inhibition was found to be strongly dependent on the concentration of GTP. Inhibition of the rate of polyphenylalanine synthesis by cryptopleurine was inversely related to the GTP concentration. Double-reciprocal plots of these data are shown in Figure 1. The lines obtained in the absence or presence of cryptopleurine intersected within experimental variation on the vertical axis. Similar experiments with mutant ribosomes also suggested competive inhibition (data not shown).

The apparent competition between cryptopleurine and GTP suggested a possible mechanism for cryptopleurine resistance. If the affinity of ribosomes for GTP were increased by a mutational alteration, then, presumably, cryptopleurine would not be as effective an inhibitor. Using the kinetic data described above, we determined the apparent $K_m$ of GTP for polyphenylalanine synthesis with both parental and mutant ribosomes. In both cases, a value of $3 \times 10^{-5}$ M was obtained, suggesting that the affinity for GTP was not altered in the mutant ribosomes. Of course, the $K_m$ determined for polyphenylalanine synthesis may not reflect the affinity of ribosomes for GTP; direct binding studies are needed to unequivocally answer this question.

The kinetic results shown in Figure 1 suggested that cryptopleurine might inhibit a GTP-requiring step of polyphenylalanine synthesis. However, because of the complexity of this system, we wondered whether such a straightforward conclusion could be drawn. In order to judge the validity of the kinetic data, we tested other inhibitors with more fully characterized mechanisms. Fusidic acid, which inhibits protein synthesi by preventing the dissociation of a ribosome-EF-2-GDP complex (Bodley et al., 1969), was competitive; anisomycin, which inhibits the peptide bond forming step (Neth et al., 1970), was noncompetitive. In these two cases, the kinetic results were consistent with the known mechanisms of inhibition. We suggest that the competitiveness observed between GTP and cryptopleurine means that cryptopleurine affects a GTP-requiring step in polyphenylalanine synthesis.

Two separate steps of elongation, the EF-1 dependent binding of aminoacyl-tRNA and the EF-2 dependent translocation of peptidyl-tRNA, have been shown to require GTP (see Haselkorn and Rothman-Denes, 1973, for review). The model implies that GTP binding to ribosomes is mediated through EF-1 or EF-2. If a ribosome-GTP complex were resistant to cryptopleurine, the complex would also contain an elongation factor. The yeast system used in these studies required a third protein in addition to ribosomes, EF-1, and EF-2. Partially purified preparations of this protein (EF-3) have been previously shown to possess ribosome dependent GTPase activity (Skogerson and Wakatama, 1976). The function of this new protein is not yet known. As shown in Figure 2, the inhibition of polyphenylalanine synthesis by $10^{-6}$ M cryptopleurine was significantly greater when rate-limiting (compared to saturating) amounts of either EF-2 or EF-3 were used. The concentration of EF-1 had no effect on the degree of inhibition. These results raised the possibility that the protection from cryptopleurine inhibition by GTP also required the presence of EF-2 and EF-3.

Kinetic experiments described above indicated that mutation to cryptopleurine resistance did not affect the $K_m$ for GTP. The

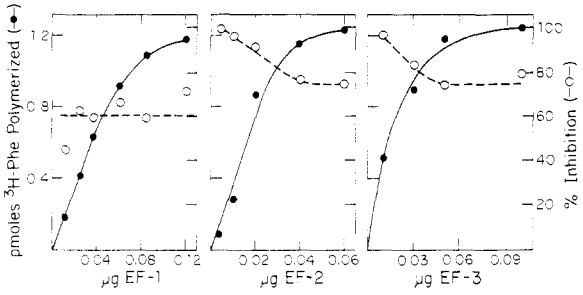


FIGURE 2: Effect of elongation factor concentrations on inhibition by cryptopleurine. Rates of polyphenylalanine synthesis were measured as a function of each elongation factor in the absence (●) or presence of $10^{-5}$ M cryptopleurine. Percent inhibition was determined for each point (○).

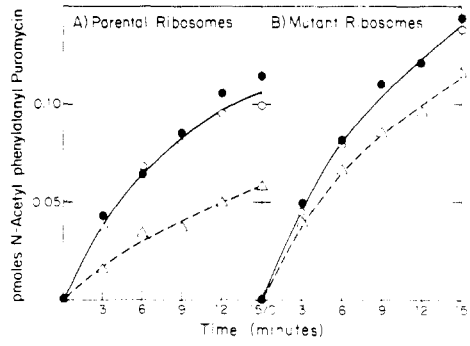


FIGURE 3: Effect of cryptopleurine on *N*-AcPhePuro synthesis. Synthesis of *N*-AcPhePuro was carried out in a three-stage reaction sequence as described in the text. Cryptopleurine was either absent (●), added for the translocation step (△), or added for the peptidyl transfer step (○). *N*-AcPhePuro was determined at the indicated times after addition of puromycin.

observation that the degree of inhibition with sensitive ribosomes was a function of EF-2 and EF-3 concentrations raised the question of whether the mutation to resistance might be expressed through altered EF-2 or EF-3 ribosomal interaction. Experiments similar to those described by Figure 2 were performed with mutant ribosomes. Qualitatively, the results were similar. At low concentrations of EF-2 and EF-3, inhibition was greater. With saturating factors, the normal mutant level of inhibition was observed (data not shown). As in Figure 2, EF-1 concentration had no effect on inhibition. Although the response of ribosomes to cryptopleurine was affected by GTP and factors, resistance did not appear to be mediated through the interaction of GTP, EF-2 or EF-3 with ribosomes. Nevertheless, these observations suggest that cryptopleurine is more likely an inhibitor of translocation than binding.

Using the binding assay described under Methods, we tested for inhibition of this reaction directly. Both the rates of (0.15 pmol/min) and extent (1.05 pmol) of binding were unaffected by $10^{-6}$ M cryptopleurine. This concentration of cryptopleurine would have inhibited polyphenylalanine synthesis 50–60% (Table I). We conclude that cryptopleurine does not directly affect the binding reaction.

Among the various ways of measuring translocation independently of polypeptide synthesis is the translocation-dependent formation of *N*-acetylphenylalanylpuromycin (Siler and Moldave, 1969). This model was particularly useful in these studies, since both translocation and peptidyl transfer are required, and can be separated experimentally. Following the binding of *N*-AcPhe-tRNA in the presence of 24 mM $MgCl_2$, the components required for translocation were added in the presence or absence of $10^{-6}$ M cryptopleurine. In this experiment, the rate of translocation was limited by the concentration of EF-2. Subsequent to translocation puromycin was added, with or without cryptopleurine, and the rate of *N*-AcPhePuro formation was measured. As seen in Figure 3A, the presence of cryptopleurine during translocation inhibited the initial rate of *N*-AcPhePuro formation about 40%, but had no effect when added after translocation was accomplished. When a similar experiment was carried out with mutant ribosomes, little inhibition of the initial rate was observed, regardless of when cryptopleurine was added (Figure 3B). The difference observed between parental and mutant ribosomes in their response to cryptopleurine in this reaction supports the idea that translocation is the stage of protein synthesis inhibited by cryptopleurine.

## Discussion

According to the two-site model for ribosome function, elongation factors mediate GTP binding to two distinct ribosomal states (see Haselkorn and Rothman-Denes, 1973, for review). A ternary complex of EF-1, aminoacyl-tRNA, and GTP binds to ribosomes in which peptidyl-tRNA occupies the P site (posttranslocation). Following peptide bond formation, a new ribosomal state is created in which peptidyl-tRNA occupies the A site and a deacylated tRNA the P site (pretranslocation). EF-2 and GTP bind to ribosomes in this state to initiate the complicated translocation process. The observation that EF-2 and GTP antagonize cryptopleurine inhibition in an apparently competitive manner suggests that cryptopleurine binds to ribosomes in the pretranslocation state. If GTP and cryptopleurine binding are truly competitive then translocation should be the inhibited step. Huang and Grollman (1972) reported that tylocrebrine, another phenanthrene alkaloid, increased the binding of deacylated tRNA to rabbit reticulocyte ribosomes. They suggested that tylocrebrine might inhibit translocation by preventing the release of the deacylated tRNA. A similar mechanism for cryptopleurine would be consistent with our finding that cryptopleurine inhibited the translocation phase of *N*-AcPhePuro synthesis. In this assay, *N*-AcPhe-tRNA was bound to ribosomes in the presence of 24 mM $MgCl_2$. Although we have not yet analyzed the complex for the presence of deacylated tRNA, we expect that the ribosomes should be in the pretranslocated state with deacylated $tRNA^{Phe}$ in the P site and *N*-AcPhe-tRNA in the A site. This complex then is the species to which either cryptopleurine or EF-2 and GTP could bind In fact, cryptopleurine inhibition of *N*-AcPhePuro synthesis was more sensitive to excess EF-2 than polyphenylalanine synthesis (data not shown).

Unfortunately, the site of cryptopleurine binding could not be determined directly because of the large degree of nonspecific binding. The equilibrium binding data may explain why cryptopleurine inhibited peptidyl transfer at high concentrations. Pestka et al. (1972) showed that $10^{-4}$ M cryptopleurine inhibited peptidyl-puromycin formation by 40%. At this high concentration, enough cryptopleurine could have bound to, perhaps, inhibit the reaction. The observation that $10^{-6}$ M cryptopleurine inhibited translocation on parental ribosomes, but not mutant ribosomes, suggests that the in vivo site of inhibition at low concentrations is translocation.

Both GTP and EF-2 interaction with ribosomes are known to be mainly determined by the larger ribosomal subunit. Questions regarding the location of the binding site for cryptopleurine and the nature of the ribosomal alteration which overcomes inhibition have yet to be answered. Does cryptopleurine bind to the 40S subunit and affect a 60S site, or does a 40S alteration affect a 60S cryptopleurine binding site?

Spectinomycin has been reported to inhibit translocation with *Escherichia coli* ribosomes (Davies et al., 1967; Burns et al., 1973), and resistant mutants have an altered 30S ribosomal protein, S-5 (Bollen et al., 1969; Funatsu et al., 1972). This system may be analogous to that of cryptopleurine inhibition with 80S ribosomes.

The newly proposed model for ribosome structure, determined by immunoelectron microscopy, depicts a high degree of interaction between the 30S and 50S subunits (Tischendorf et al., 1975). If that model is basically correct, the interactions are such that a number of 30S proteins could be in contact with the area on the 50S where the EF's and GTP interact. The possible mechanisms for cryptopleurine resistance and inhibition could easily be accommodated by such a model.

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