PUROMYCIN INHIBITION OF PROTEIN SYNTHESIS: INCORPORATION OF PUROMYCIN INTO PEPTIDE CHAINS

BY DANIEL NATHANS

DEPARTMENT OF MICROBIOLOGY, THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE

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The similarity in structure between puromycin and aminoacyl-sRNA (Fig. 1), first noted by Yarmolinsky and de la Haba,[1] led to the hypothesis that the antibiotic inhibits protein synthesis by acting as an analogue of esterified sRNA. Puromycin blocks protein synthesis after aminoacyl-sRNA formation,[1, 2] and at the same time it leads to the accumulation of small peptides.[3] Both of these effects appear to be due to the splitting of ribosome-bound peptidyl-sRNA,[4] which results in release of incomplete peptide chains.[5–7] If puromycin does act as an analogue of "charged" sRNA, one might anticipate the possibility that it could substitute for an incoming aminoacyl-sRNA as the acceptor of the carboxyl-activated peptide, forming peptidyl-puromycin, thus ending growth of the polypeptide.[7, 8] Such a mechanism is supported by the finding of Allen and Zamecnik[7] that when puromycin labeled in the amino acid moiety is incubated with reticulocyte ribosomes, a portion of the radioactivity precipitates with the released polypeptides, the amount being equivalent to the N-terminal valine of these peptides. In order to establish, however, that puromycin replaces aminoacyl-sRNA as an acceptor of the peptide chain it is desirable to show unequivocally that puromycin as such becomes linked to polypeptide via a peptide bond in the course of protein synthesis. The study reported here was undertaken to determine whether or not peptidyl-puromycin is formed when susceptible cells are exposed to the antibiotic.

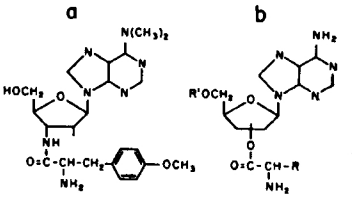


FIG. 1.—The structure of puromycin (*a*) and of aminoacyl-sRNA (*b*).

Materials and Methods.—H^3-puromycin, labeled in the methoxy group, was prepared under the

guidance of Dr. Amos Neidle of Columbia University, New York, by first methylating N-carbobenzoxy-L-tyrosine (Mann Research Laboratories, New York, N. Y.) with H^3-dimethyl sulfate (120 mc/mmole, New England Nuclear Corp., Boston, Mass.) according to the procedure of Pitt-Rivers and Lerman.[9] The resulting H^3-N-carbobenzoxy-O-methyl-L-tyrosine was then condensed with the aminonucleoside of puromycin (obtained from Dr. Leon Goldman of the Lederle Laboratories, Pearl River, N. Y.), following the procedure of Baker *et al.*[10] to give H^3-N-carbobenzoxy-puromycin (mp 193–195°). H^3-N-carbobenzoxypuromycin was reduced with hydrogen in the presence of palladium oxide, and the resulting H^3-puromycin was crystallized from alcohol (mp 160–163°).

The product was analyzed by paper chromatography in butanol-acetic acid-water (4:1:5) and by paper electrophoresis at pH 4.7 in pyridine acetate. In each case a single ultraviolet-absorbing spot was found corresponding with puromycin and containing 90–93% of the recoverable radioactivity. Ultraviolet absorption spectra of the H^3-puromycin corresponded closely with that of puromycin; wavelengths giving maximal and minimal absorption at pH 1, 7, and 11 were the same as with puromycin. Prior to use in the experiments to be described the H^3-puromycin was further purified by paper electrophoresis.

L-phenylalanyl-puromycin (9-(3-deoxy-3-L-phenylalanyl-O-methyl-L-tyrosinamido-β-D-ribofuranosyl)-6-dimethylamino-9H-purine) was prepared by condensing N-carbobenzoxy-L-phenylalanine (Mann Research Laboratories) with puromycin.[10] Glycyl-puromycin (9-(3-deoxy-3-glycyl-O-methyl-L-tyrosinamido β-D-ribofuranosyl)-6-dimethylamino-9H-purine) and puromycin were supplied by Dr. Goldman. O-methyl-L-tyrosine was obtained by the reduction of N-carbobenzoxy-O-methyl-L-tyrosine prepared as described above. Chymotrypsin, trypsin, and carboxypeptidase A (treated with diisopropylfluorophosphate) were purchased from Worthington Biochemical

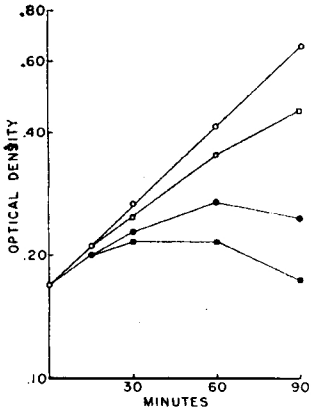


FIG. 2.—The effect of different concentrations of puromycin on the growth of *E. coli*. Aliquots of a log-phase culture were continuously aerated in bubbler tubes at 37° and samples removed at various times for optical density readings at 600 mμ. Puromycin concentrations were as follows: zero (○), 3.2×10^{-5} *M* (□), 8×10^{-5} *M* (●), 1.6×10^{-4} *M* (■). In other experiments no fall in optical density was detected during 2 hr of growth in the presence of 8.0×10^{-5} *M* or 1.6×10^{-4} *M* puromycin.

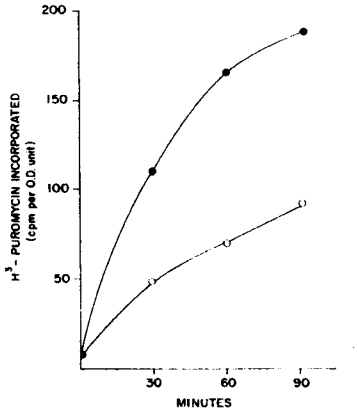


FIG. 3.—Time course of H^3-puromycin incorporation into an acid-precipitable product. To 2 ml aliquots of a logarithmic phase culture (O.D. of .565) H^3-puromycin (1.6×10^6 cpm/μmole) was added to give a final concentration of 3.2×10^{-5} *M* (○) or 8.0×10^{-5} *M* (●). At various times during growth at 35° 0.4-ml samples were removed, and carrier protein and excess unlabeled puromycin were added, followed by an equal volume of 10% trichloroacetic acid (TCA). The precipitate was dissolved in NaOH, reprecipitated with 5% TCA in the presence of unlabeled puromycin, washed with TCA and ether, and counted. Separate aliquots of the original culture were used for following the optical density. At the end of 90 min the optical density was 1.74 in the absence of puromycin, 1.61 with 3.2×10^{-5} *M* puromycin, and 1.02 with 8.0×10^{-5} *M*. Incorporation is given as cpm per 0.4 ml of culture divided by the optical density expressed in multiples of the zero time value.

Corp., Freehold, N. J.; and pronase, from Calbiochem, Los Angeles, Cal. Each enzyme was active with model substrates.

E. coli B used in these experiments was grown in a minimal medium of the following composition: K_2HPO_4 10.5 gm, KH_2PO_4 4.5 gm, sodium citrate·$5H_2O$ 0.47 gm, $(NH_4)_2SO_4$ 1.0 gm, $MgSO_4 \cdot 7H_2O$ 10 mg, and glucose 2.0 gm per liter of water. A low magnesium ion concentration was used since the sensitivity of *Pseudomonas fluorescens* to puromycin had been reported to increase as the magnesium ion concentration of the medium was lowered.[11] A similar effect was found with *E. coli*. At the magnesium level used in the present experiments, growth was the same as at higher concentrations.

Radioactivity measurements were carried out in a liquid scintillation counter, using a toluene-based scintillator.

Results.—Incorporation of H^3-puromycin into an acid-precipitable product: At concentrations of puromycin which inhibit growth of *E. coli* (Fig. 2) and amino acid incorporation into protein, H^3-puromycin is incorporated into an acid-precipitable product. As shown in Figure 3, this reaction is time- and concentration-dependent; with the concentrations used, the amount of puromycin incorporated per unit cells is proportional to concentration. On dilution of H^3-puromycin with nonradioactive puromycin the amount of radioactivity in the acid-precipitate is reduced proportionately; in contrast, addition of a thirtyfold molar excess of nonradioactive phenylalanine and tyrosine to the medium did not affect the amount of radioactivity incorporated (Table 1). These results suggest that

TABLE 1

Puromycin ($M \times 10^5$)	Specific activity (cpm $\times 10^{-6}$/μmole)	Additions	Puromycin incorporated cpm	Puromycin incorporated mμmoles
Exp. 1				
3.2	3.20	—	2040	0.64
3.2	3.20	Chloramphenicol	90	0.028
8.0	3.20	—	2490	0.78
8.0	1.28	—	1070	0.83
Exp. 2				
3.2	1.28	—	1560	1.22
3.2	1.28	Tyrosine and phenylalanine	1510	1.18

The effect of chloramphenicol, unlabeled puromycin, and unlabeled tyrosine and phenylalanine on the incorporation of H^3-puromycin into an acid-precipitable product. One-ml aliquots of a logarithmic phase culture (O.D. .400 in exp. 1 and .750 in exp. 2) were grown at 37° for 90 min in the presence of the compounds indicated. Chloramphenicol was added 5 min prior to puromycin at a concentration of 50 μgm/ml. L-tyrosine and L-phenylalanine were added 15 min prior to puromycin at concentrations of 0.001 *M* each. The samples were analyzed as described in the legend of Fig. 3.

the incorporated radioactivity is not due to adsorption nor to splitting of the amide bond of puromycin with the subsequent incorporation of O-methyl-tyrosine into protein. Also, the fact that a puromycin-resistant mutant of *E. coli* B, when grown for 90 min in the presence of 8×10^{-5} *M* H^3-puromycin, showed only 13 per cent as much incorporation as the wild-type organism suggests that the incorporation is related to the inhibitory effect of the antibiotic. Furthermore, the marked inhibition of puromycin incorporation by chloramphenicol (Table 1) indicates a relationship between this reaction and protein synthesis and is similar to the effect of chloramphenicol on the production of puromycin-induced peptides in the aminoacyl-sRNA-ribosomal system.[8]

Identification of the acid-insoluble product as polypeptide: A larger scale incubation of log-phase cells with 3.2×10^{-5} *M* H^3-puromycin (specific activity 1.14×10^6 cpm/μmole) was carried out for 90 min at 37°, and the cold TCA precipitate,

after being washed with five per cent TCA and extracted with ether, was redissolved and digested with RNAse and DNAse followed by prolonged dialysis. (With this procedure a zero-time sample showed about two per cent as much acid-precipitable radioactivity as the 90-min sample.) Aliquots of the nondialyzable labeled material were digested with various proteolytic enzymes or with 6 *N* HCl and the digests chromatographed on paper in butanol-acetic acid-water (4:1:5). Segments of the paper were then counted for radioactivity. The results are shown in Figure 4. In the absence of any proteolytic agent almost all the radioactivity remained at the origin. HCl and pronase treatment liberated radioactive O-methyl-tyrosine

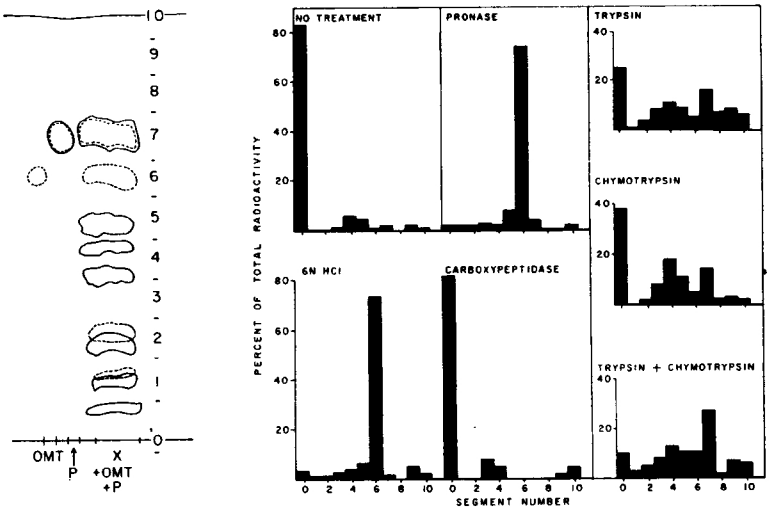


FIG. 4.—The effect of proteolytic agents on the chromatographic distribution of radioactive material. Aliquots of the acid-precipitable H^3-puromycin product, prepared as described in the text, were incubated with the agents indicated. In the case of the control ("no treatment") and the proteolytic enzymes, incubations were for 3 hr at 35°. Acid hydrolysis was carried out at 100° for 18 hr in 6 *N* HCl. Puromycin (P) and O-methyl-tyrosine (OMT) were added to each sample prior to chromatography. On the left is a tracing of the chromatogram of one of the samples. Unbroken lines outline ultraviolet-absorbing spots; broken lines outline ninhydrin-staining spots. The top line represents the solvent front. The chromatogram was cut into segments as indicated by the numbers, and each segment counted. On the right is plotted the percentage of total counts found in each segment. Total radioactivity was approximately 1000 counts per minute in each case.

suggesting that this amino acid was in peptide linkage. Separate or combined trypsin and chymotrypsin digestions led to many different radioactive spots, again indicating that the radioactive material is part of peptide chains. As shown in Figure 4, incubation with carboxypeptidase had no effect on the chromatographic distribution of radioactivity. The fact that carboxypeptidase treatment did not liberate radioactive O-methyl-tyrosine suggests that this amino acid was not present as such at the carboxyl end of the peptide chains. Furthermore, no O-methyl-tyrosine was found even after carboxypeptidase treatment of radioactive peptides formed by combined trypsin-chymotrypsin digestion. This result suggests that

the O-methyl-tyrosine moiety of puromycin was not incorporated into the peptide product as the free amino acid.

Identification of puromycin in the polypeptide chains: Both trypsin and chymotrypsin digestion of the radioactive acid-precipitable product, and especially combined trypsin-chymotrypsin treatment, yielded radioactive peaks on chromatography corresponding in mobility to puromycin (Fig. 4). In view of the apparent

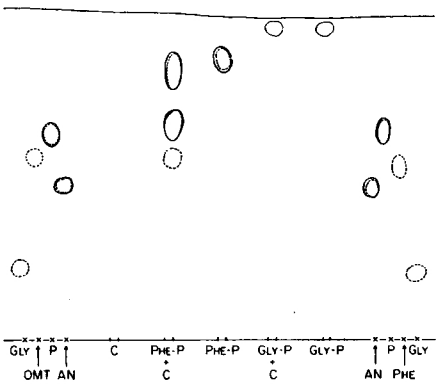


FIG. 5.—Chromatogram of chymotrypsin digests of phenylalanyl-puromycin and of glycyl-puromycin. Conditions for incubation and chromatography were the same as indicated in the legend of Fig. 4. *Gly*, glycine; *OMT*, O-methyl-tyrosine; *P*, puromycin: *AN*, aminonucleoside of puromycin; *C*, chymotrypsin; *Phe-P*, phenylalanyl-puromycin; *Gly-P*, glycyl-puromycin; *Phe*, phenylalanine. Unbroken lines outline ultraviolet-absorbing spots; broken lines outline ninhydrin-staining spots.

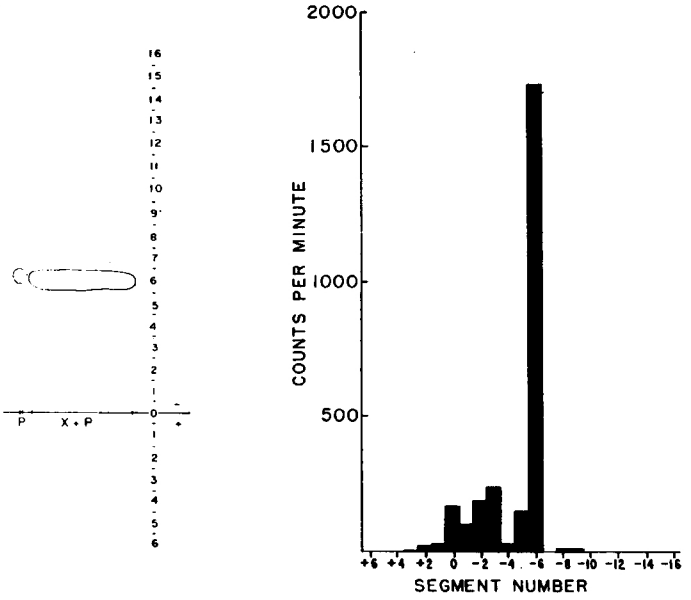


FIG. 6.—Electropherogram of radioactive material liberated by trypsin-chymotrypsin digestion and having the mobility of puromycin on chromatography. Electrophoresis was carried out at pH 4.5 in pyridine-acetic acid-butanol (2.5, 2.5, 5% v/v) on Whatman 3 MM paper at 30 V/cm for 2.5 hr. On the left is a tracing of the ultraviolet-absorbing spot corresponding to added puromycin (*P*). *X* represents the radioactive material. On the right are plotted the counts per minute in the various segments of paper.

liberation of puromycin by chymotrypsin and the results with carboxypeptidase mentioned above, it appeared that the amide bond of puromycin was resistant to chymotrypsin. To demonstrate this more directly the action of chymotrypsin on synthetic aminoacy-puromycins was examined.

As shown in Figure 5, when phenylalanyl-puromycin or glycyl-puromycin was treated with chymotrypsin, no aminonucleoside was formed, i.e., the amide bond between O-methyl-tyrosine and the 3′ amino group was not split. With phenylalanyl-puromycin, phenylalanine and puromycin were found. Therefore, chymotrypsin would be expected to liberate puromycin from a polypeptidyl-puromycin where the last amino acid of the polypeptide is phenylalanine or tyrosine. Similarly (though this was not directly tested), trypsin should lead to free puromycin wherever lysine or arginine is the last amino acid of the polypeptide chain.

In order to identify the radioactive compound(s) liberated by trypsin-chymotrypsin digestion of the H^3-puromycin product and moving with puromycin on chromatography, this material was eluted and electrophorized. As shown in Figure 6 about 65 per cent of the radioactivity migrated with puromycin. Moreover, when

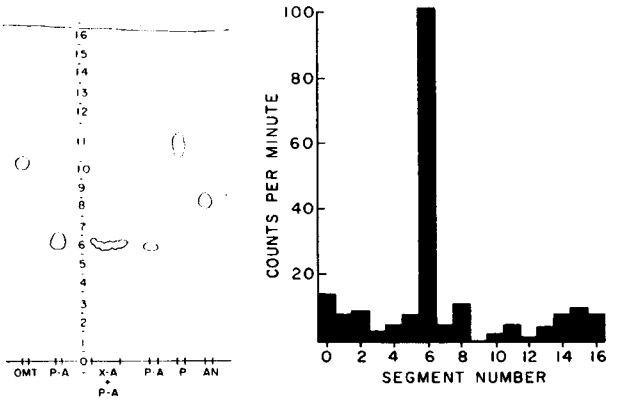


FIG. 7.—Chromatography of the acid-treated putative H^3-puromycin in butanol-acetic acid-water. On the left is a tracing of the chromatogram stained with ninhydrin. The line at the top represents the solvent front. Legends are the same as given under Fig. 5. *A* indicates acid-treated; *X*, the radioactive material. On the right are plotted the counts per minute in the various segments of paper.

an aliquot of the material purified by consecutive chromatography and electrophoresis was treated with 1 *N* HCl at 100° for 15 min to depurinate the putative puromycin, most of the radioactivity then moved on chromatography with the depurination product of puromycin (Fig. 7). Finally, the purified radioactive material was crystallized together with carrier puromycin to constant specific activity (Table 2). We conclude therefore that puromycin is incorporated as such into the polypeptide chain of protein by means of a peptidase-sensitive bond. Since the molecule has no carboxyl or similar group capable of continuing the chain, it must be present at the carboxyl terminal end of the polypeptide.

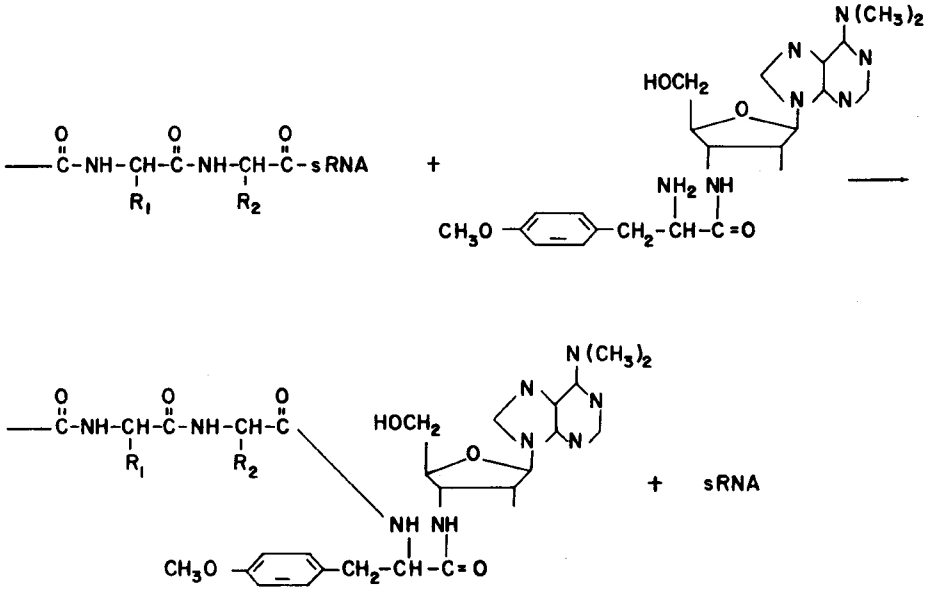
Discussion.—The finding of puromycin within (and therefore at the end) of

TABLE 2

	O.D. units	Cpm	Cpm per 1000 O.D. units
Starting material	1760	697	396
2nd crystallization	388	102	263
3rd crystallization	510	128	251
4th crystallization	329	92	279

To an alcoholic solution of the material purified by consecutive chromatography and electrophoresis (as described in the text) 87 μmoles of puromycin dihydrochloride were added, followed by an equivalent amount of cyclohexylamine. The puromycin was dissolved at 65–70° and allowed to crystallize overnight at 4°. The crystals were washed with alcohol at −15°, redissolved in warm alcohol, and aliquots taken for counting and measurement of O.D. at 267 mμ. This crystallization procedure was repeated three times. The product of the first crystallization was not analyzed.

polypeptide chains can readily account for the inhibition of protein synthesis by this antibiotic. Whereas peptidyl-sRNA found on the ribosome[4] is ordinarily transferred to the amino group of the next aminoacyl-sRNA, in the presence of puromycin the carboxyl-activated peptide appears to be transferred to puromycin, thus ending the sequential extension of the protein chain:



Although this mechanism can account for the inhibition of protein synthesis by puromycin, it is not clear that this is the only way in which it functions. In the aminoacyl-sRNA-ribosomal system, puromycin leads to the formation of small peptides without puromycin at the end.[3] (Such peptides would not have been detected in the present study.) The appearance of free peptides suggests that puromycin may bring about hydrolytic cleavage of the peptidyl-sRNA bond in addition to the transfer mechanism already discussed. However, because the observations on formation of free peptides were made under conditions where peptidases might secondarily split peptidyl-puromycin,[3] this alternate reaction is not established.

An implication of the mode of action of puromycin presented here is the likelihood that the transfer of peptide from peptidyl-sRNA to puromycin can serve as a model for the formation of peptide bonds in protein synthesis. Thus, one would expect that the same enzyme that catalyzes peptide bond formation also catalyzes the transfer of peptide to puromycin. In line with this is the observation that release of peptides from *E. coli* ribosomes by puromycin is stimulated by the enzyme fraction which is required for polypeptide synthesis from aminoacyl-sRNA.[12]

Summary.—Radioactive puromycin, when added to a culture of *E. coli*, becomes incorporated into acid-precipitable compounds by means of a reaction that is sensitive to chloramphenicol. Digestion of this acid-insoluble product with trypsin and chymotrypsin liberates radioactive material with chromatographic, electrophoretic, and chemical properties of puromycin. It is concluded that growing peptide chains on ribosomes are transferred to the free amino group of puromycin in analogy with the normal reaction of peptide bond formation in protein synthesis.

The generous assistance of Dr. Amos Neidle in the synthesis of radioactive puromycin and of phenylalanyl-puromycin is most gratefully acknowledged, as is the continued interest of Dr. Fritz Lipmann, in whose laboratory the author's work on puromycin was begun. This research has been supported by a grant from the National Institutes of Health.

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