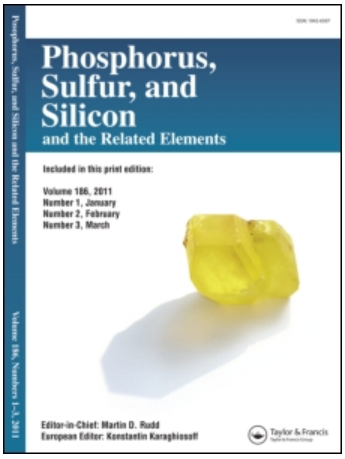

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ORGANOPHOSPHORUS COMPOUNDS AS ANTIVIRAL AGENTS

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The 5′-triphosphates of 2′-5′ linked oligoadenylic acids are formed in cells which have been exposed to interferon and may be involved in the antiviral activity of the latter. The lead(II) ion-catalysed oligomerisation of adenosine 5′-phosphorimidazolidate is a convenient route for the preparation of the 5′-phosphates of 2′-5′ linked oligoadenylic acids. The latter can readily be converted to the triphosphates or coupled to the 5′-phosphate of nicotinamide nucleoside to give naturally occurring pyrophosphates which may act as reservoirs for the oligoadenylic acids in cells.

Pyrophosphate analogues, eg, phosphonoacetic and phosphonoformic acids or carbon-substituted methylenebisphosphonic acids are antiviral agents of potential commercial interest as they inhibit the replication of a number of viruses including herpes and influenza. These pyrophosphate analogues do not appear to inhibit virus replication by being incorporated into nucleoside triphosphates which block nucleic acid synthesis. Rather the analogues appear to act by forming stable complexes with an essential metal ion (probably zinc) at the active sites of nucleic acid polymerases of viruses.

At the present time, most bacterial infections can be controlled by chemotherapeutic agents as many bacterial enzymes are unlike those of the host organism. On the other hand, the enzymes of viruses are often very similar to those of the host organism and hence the chemotherapeutic control of viruses is difficult. There are, however, some small differences between host and viral enzymes and these have been exploited recently in a number of laboratories. Particular progress in the chemotherapy of virus infections has been made in three areas: (a) nucleoside analogues, (b) interferon, and (c) low molecular weight inhibitors of viral enzymes. Nucleoside analogues are outside the scope of this review but I would like to consider briefly some progress which is being made in the interferon field before concentrating on the low molecular weight inhibitors.

Interferon is a naturally occurring glycoprotein which can confer antiviral properties on cells. When interferon acts on susceptible cells, the synthesis of some unusual oligonucleotides, eg, the 5′-triphosphate of adenylyl(2′-5′)adenylyl(2′-5′)adenosine (1) and higher oligomers is enhanced. This oligonucleotide is unusual as it is the only naturally occurring nucleic acid derivative to be discovered at the present time which contains 2′-5′ rather than the more normal 3′-5′ internucleotide links. These oligomers have a powerful inhibitory effect on protein synthesis in cells even when they are present at very low concentrations. One important sequence of events which is triggered within the cell by (1) is the phosphorylation of a nuclease and this can then

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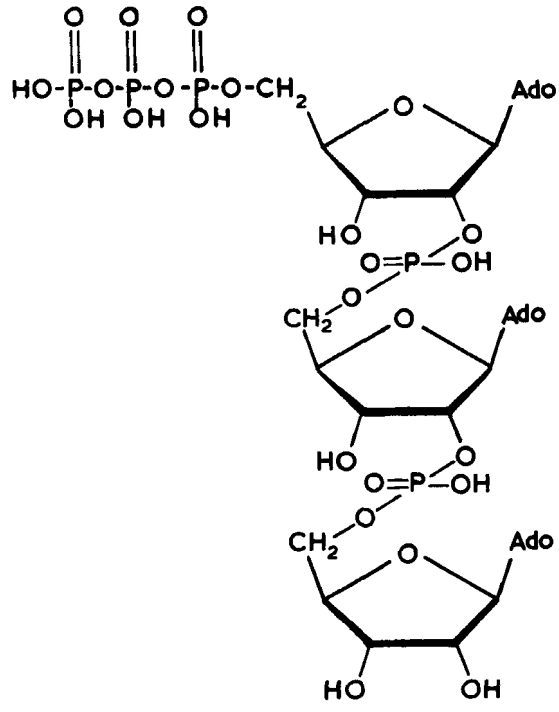


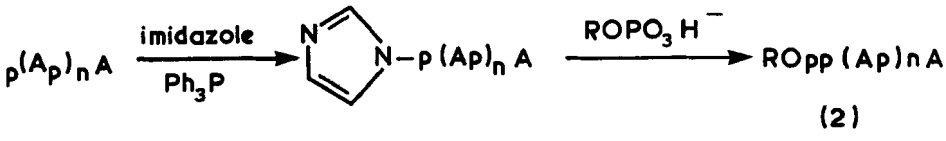
(1)

degrade single stranded RNA. As the mRNA of viruses is single stranded the activated nuclease prevents viral replication.

Thus, the oligonucleotide (1) would seem to have potential as an antiviral agent. However, at least three problems have to be overcome before (1) can be used. These are (a) the synthesis of substantial amounts of the oligonucleotides, (b) the poor uptake of (1) by the cells and (c) their low stability in cells. There are some twenty syntheses of (1) in the literature[1] but most of these are complex, multistage procedures involving protecting groups and the overall yields are low. An attractive method for the synthesis of (1) is based on prebiotic models for oligonucleotide synthesis.[2] Oligomerisation of the 5′-phosphorimidazolidate of adenosine in the presence of lead(II) ions gives the 5′-phosphates of predominantly 2′-5′ linked oligoadenylic acids. Some 3′-5′ linked oligomers are also formed but these are easy to remove by enzymic hydrolysis. No protection of the sugar hydroxyls or the adenine residue are required and reasonable yields of the 5′-phosphates of the 2′-5′ linked oligoadenylic acids can be obtained in only a few days. The latter can then be converted into the corresponding 5′-triphosphates via their phosphoromorpholidates. The oligoadenylic acids are highly charged and are not taken up readily by cells. Various methods have been used to overcome this problem, the most successful appears to be precipitation as the calcium salts.[3] This is feasible as a research technique but obviously presents problems for *in vivo* work. Even though the oligoadenylic acids related to (1) seem to be unique in possessing 2′-5′ internucleo-

tide links and hence must have a very different shape to the 3′-5′ linked isomers, there are enzymes in cells which recognise these compounds and degrade them in minutes. One way to stabilise (1) is to block the 2′,3′-hydroxyl groups by alkylation and this aspect is under investigation in a number of laboratories. We have been intrigued by the isolation of a nicotinamide derivative (2) of the oligoadenylic acid trimer.[4] The biological function of (2) is not known but it has been suggested that it may act as a reservoir for (1) which can be formed by hydrolysis and further phosphorylation. As (2) only occurs in minute amounts in cells we have devised a synthesis based on the coupling of nicotinamide ribonucleoside 5′-phosphate with the 5′-phosphorimidazolidates of 2′-5′ linked oligoadenylic acids prepared by the lead(II) ion-catalysed method.

The biological properties of (2) are being investigated at the Imperial Cancer Research Foundation in London.



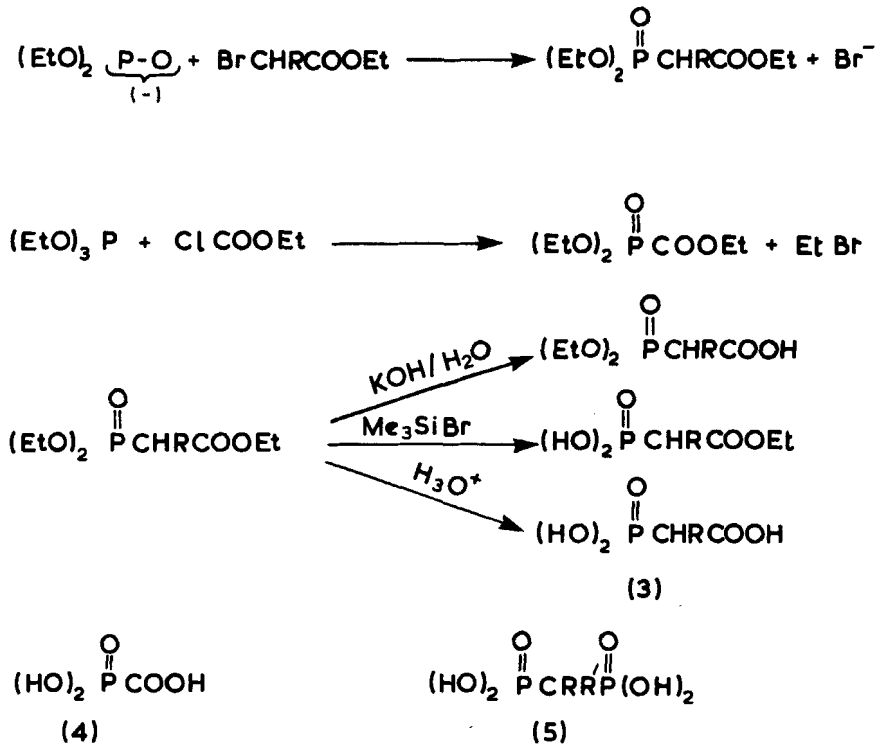
(2)

where R = nicotinamide –1–(3-β-D-ribofuranosyl)5′

Although oligoadenylic acids related to (1) have antiviral properties under suitable conditions, problems outlined above associated with production, uptake and stability do not make them promising antiviral agents for clinical use at the moment. Low molecular weight inhibitors of viruses are more attractive commercially as they should be simple compounds and hence comparatively easy to manufacture. Moreover, it should be possible to introduce variations in structure by synthetic methods to overcome problems of uptake, toxicity, stability, etc., without undue difficulty.

One promising class of low molecular weight inhibitors of virus replication which we have been studying are analogues of pyrophosphoric acid. Examples of these analogues, phosphonoacetic (3, R = H) and phosphonoformic (4) acids have been known for many years and they can be prepared either by the Michaelis Becker[5] or Arbusov reactions.[6] Since (3) and (4) contain both carboxylic and phosphoric acid residues it is a simple matter to prepare derivatives modified at either residue.[7,8] Another class of pyrophosphate analogues which we have found to be effective antiviral agents are carbon-substituted methylenebisphosphonic acids (5).

We have prepared a number of derivatives of (3–5) and required a simple way to analyse our products. Nuclear magnetic resonance spectroscopy was not very useful and hence we turned our attention to mass spectrometry. Electron impact mass spectrometry was unsatisfactory as the parent molecule could not easily be detected as it carried only a very small percentage of the total ion current. Chemical ionisation mass spectrometry using ammonia as the reagent gas gave spectra in which the protonated molecular ion carried 10–30% of the total ion current.[9] Not only were intense molecular ions present but useful structural information could be obtained as only limited fragmentation of the protonated molecular ions occurs with



ammonia as reagent gas. In the ammonia chemical ionisation mass spectrum of triethyl phosphonoacetate, the $(M + H)^+$ peak appears at m/z 225 and there are peaks corresponding to the successive loss of ethylene residues (28 mass units) at m/z 197, 169 and 141 confirming the presence of a triethyl ester. The successive dealkylation of phosphate esters during ammonia chemical ionisation mass spectrometry is quite general and for example, tetraisopropyl methylenebisphosphonate shows a $(M + H)^+$ peak at m/z 345 and peaks at m/z 303, 261, 219 and 177 corresponding to the successive loss of four propylene residues (42 mass units) (Figure 1). In this manner we have been able to analyse halogenated and other derivatives of (3–5).

The pyrophosphate analogues (3–5) are effective inhibitors of virus replication (Table I).[10,11] They act by inhibiting nucleic acid synthesis and the presence of the free carboxylic acid and both phosphoryl hydroxyl groups in (3) and (4) or all phosphoryl hydroxyl functions in (5) is essential for antiviral activity. Modification of any of the acid functions in these compounds causes almost complete loss of antiviral activity. The initial discovery of the antiviral properties of (3) and (4) was carried out with herpes virus[12] but influenza virus, cytomegalovirus, and a number of other viruses, are also inhibited. Nucleic acid synthesis is inhibited by inhibition of the nucleic acid polymerases in the viruses but some nonviral polymerases are also sensitive such as DNA polymerase α of mammalian cells,[12] and reverse transcriptase (the RNA dependent DNA polymerase of certain tumour viruses).[13]

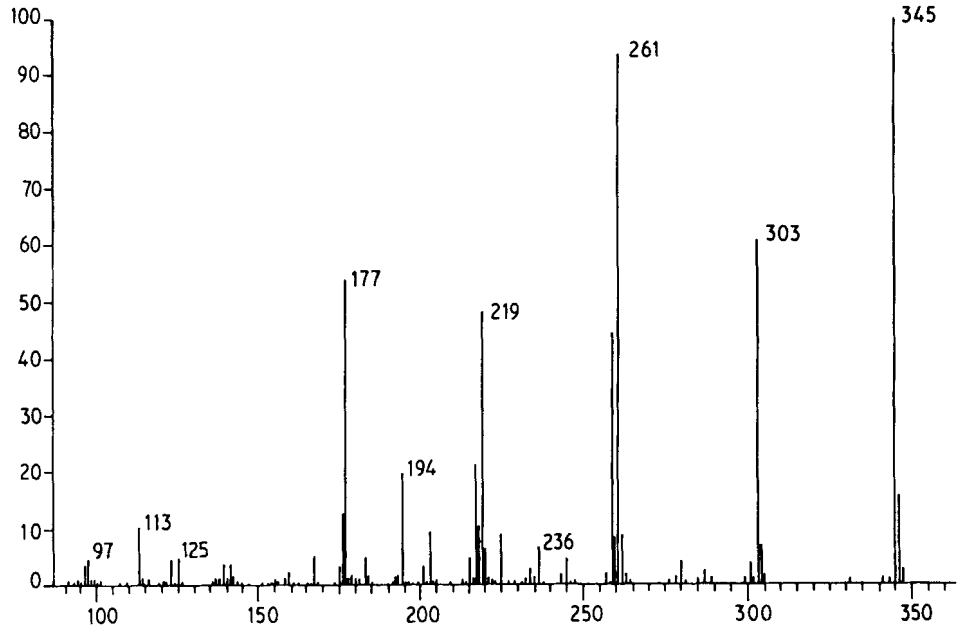


FIGURE 1 Ammonia chemical ionisation mass spectrum of tetraisopropyl methylenebisphosphonate.

We have been studying the effect of pyrophosphate analogues on influenza virus mainly because of availability and ease of handling of the virus. However, there are problems as it is difficult to isolate from influenza virus active RNA polymerase which is free from other proteins. The native nucleic acid template must be present together with an oligonucleotide promoter which makes interpretation of the enzyme kinetics of the polymerase reaction difficult. The inhibition of influenza RNA

TABLE I

Inhibitory effect of pyrophosphate analogues on RNA polymerase from influenza virus

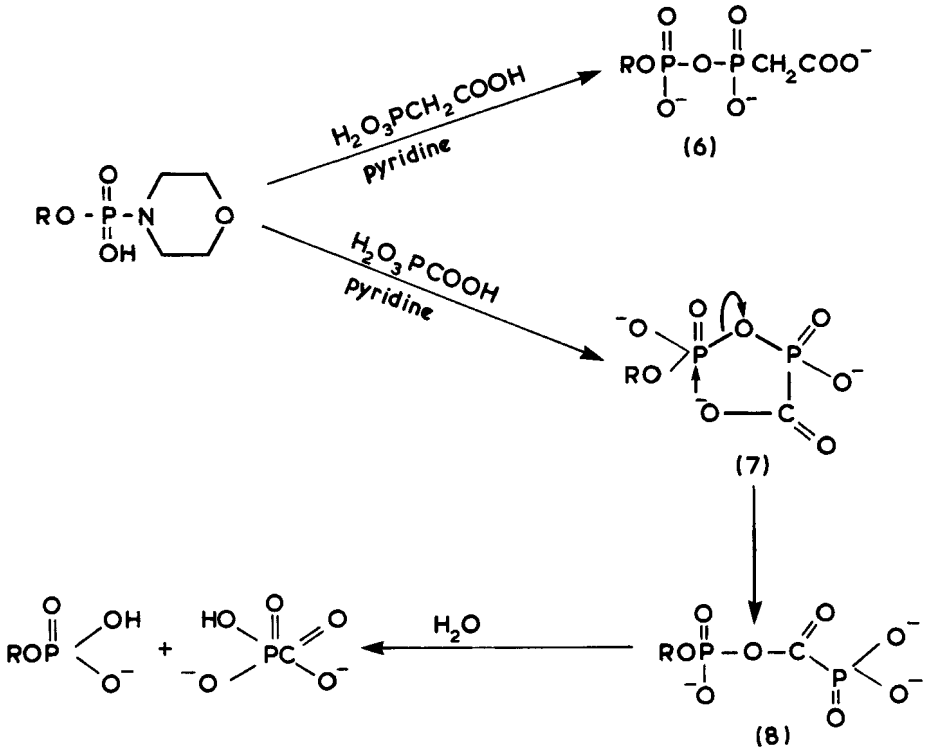
Compound	Concentration (μM) Producing 50% Inhibition of Polymerase
$(HO)_2P(O)CH_2COOH$	275
$(HO)_2P(O)COOH$	35
$(HO)_2P(O)CH_2CH_2COOH$	> 500
$(HO)_2P(O)CH_2CONH_2$	> 500
$(HO)_2P(O)OP(O)(OH)_2$	125
$(HO)_2P(O)NHP(O)(OH)_2$	50
$(HO)_2P(O)CH_2P(O)(OH)_2$	> 500
$(HO)_2P(O)CHClP(O)(OH)_2$	85
$(HO)_2P(O)CCl_2P(O)(OH)_2$	75
$(HO)_2P(O)CBr_2P(O)(OH)_2$	10
$(HO)_2P(O)COP(O)(OH)_2$	20

polymerase is followed by studying the effect of pyrophosphate analogues on the incorporation of radioactivity into acid-insoluble polynucleotide. This is more rapid and a much more sensitive assay than ones based on inhibition of viral growth.

There are two plausible mechanisms for the mode of action of the pyrophosphate analogues. Either, (a) they are converted by host or virus into analogues of nucleoside triphosphates which inhibit the viral polymerases, or (b) they interact directly with the polymerases possibly by coordinating with an essential metal ion. We do not believe that these inhibitors act by being incorporated into the β-γ positions of a nucleoside triphosphate analogue. We have prepared the ATP analogue of phosphonoacetic acid (6, R = adenosine-5′) and this compound is neither an inhibitor of nor a substrate for RNA polymerase from influenza virus. The similar thymidine analogue (6, R = thymidine-5′) is neither an inhibitor of nor a substrate for DNA polymerase from herpes virus.[16] Dichloromethylenebisphosphonic acid (5, R = R^1 = Cl) is a good inhibitor of influenza virus RNA polymerase but again its ATP analogue is neither an inhibitor of nor a substrate for the polymerase.

The ATP analogue (6, R = adenosine-5′) is easy to prepare by the phosphoromorpholidate route but when we tried to prepare the ATP analogue of phosphonoformic acid all we achieved was the rapid conversion of adenosine 5′-phosphoromorpholidate into AMP. The reaction in pyridine can be followed by ^{31}P NMR at 162 MHz using a Bruker WH400 spectrometer. With phosphonoacetic acid there was a steady decay of the phosphoromorpholidate signal at 7.8 ppm matched by an increase in intensity of a pair of doublets centred at 5.2 and −9.7 ppm due to the formation of the pyrophosphate residue in (6, R = adenosine-5′). With phosphonoformic acid, the phosphoromorpholidate signal decays as before but no new doublets can be detected, only a new singlet at 2.2 ppm due to adenosine 5′-phosphate. If the C-ethyl ester of phosphonoformic acid is used in place of the free acid, signals at 28.2 ppm due to the ester and 7.8 due to the adenosine 5′-phosphoromorpholidate can be observed at the start of the experiment. With time, two doublets centred at 20.2 and −9.4 ppm appear due to the formation of the pyrophosphate. Thus the C-ethyl ester of phosphonoformic acid which is not an inhibitor of the viral polymerase does form a nucleoside triphosphate analogue. We believe that phosphonoformic acid reacts with adenosine 5′-phosphoromorpholidate to give an ATP analogue (7) but this breaks down very rapidly in an intramolecular reaction to give a mixed anhydride of adenosine 5′-phosphoric and formic acids (8). Such mixed anhydrides are considerably more reactive than phosphoric-acetic mixed anhydrides[17] and would be expected to decompose rapidly in pyridine. With phosphonoacetic acid, the nucleoside triphosphate analogue could still decompose by an intramolecular reaction but here a six-membered rather than a five-membered ring would be involved and hence this breakdown should be comparatively slow.

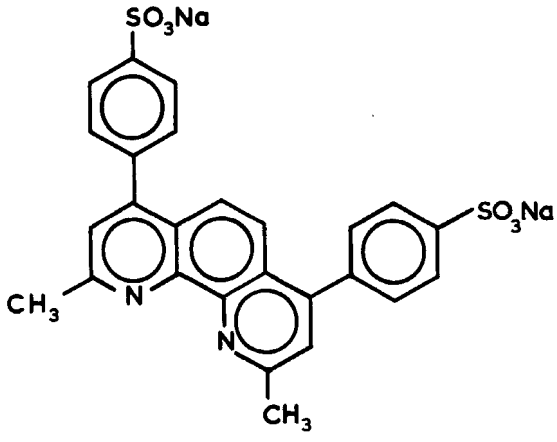
Marked differences between the rates of intramolecular hydrolysis of phosphonate esters have been observed depending on the size of the ring involved in the cyclic reaction. Thus, diethyl 2-carboxymethylphenylphosphonate undergoes intramolecular hydrolysis 10^5 times more slowly than diethyl 2-carboxyphenylphosphonate at pH 3.0 and 79.5°.[18] The C-ethyl ester of phosphonoformic acid cannot decompose via a cyclic reaction as the carboxyl group is blocked. Hence, the formation of the pyrophosphate bond in the nucleoside triphosphate analogue can be observed.



Many DNA and RNA polymerases are zinc-requiring enzymes[19,20] and we propose that phosphonoacetic, phosphonoformic and methylenebisphosphonic acids act by complexing with this zinc ion preventing the release of pyrophosphate in the chain-elongation step of the polymerisation. This proposal is not new[21] but so far little evidence has been assembled to support it. A study of enzyme kinetics using DNA polymerase from herpes virus shows that both phosphonoacetate and pyrophosphate inhibit the enzyme in an analogous manner but that pyrophosphate is the less active. Both are non-competitive inhibitors of deoxyribonucleoside triphosphates in the polymerase reaction. In addition, phosphonoacetate is a competitive inhibitor of pyrophosphate in the pyrophosphate ⇄ deoxyribonucleoside triphosphate exchange reaction which is catalysed by the polymerase.[22] This evidence suggests that phosphonoacetate blocks a site on the enzyme which binds pyrophosphate. Further support for the chelation mechanism of inhibition is the observation that bathocuproin (9) and 2-acetylpyridine thiosemicarbazone (10), both good chelating agents for "soft" metal ions such as zinc, inhibit influenza virus replication by inhibiting the RNA polymerase of the virus.[23]

Little has been published on the metal-chelating properties of pyrophosphate analogues but one study[24] does show that phosphonoacetate chelates zinc strongly.

We are investigating the metal chelating properties of our antiviral compounds and hope to see a correlation between the stability constants of the zinc complexes with antiviral activity.



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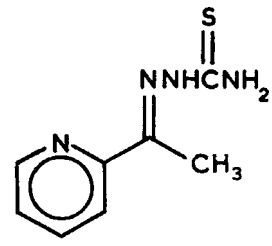

(10)

Thus, from in vitro studies, pyrophosphate analogues appear to have promise as chemotherapeutic agents. Those simple derivatives tested so far appear to suffer from one drawback. Since they are good chelators of divalent metal ions they also complex strongly with calcium ions and are deposited in the bone. The methylene-bisphosphonate (5, R = R′ = Cl) has been proposed for the treatment of Paget's disease of the bones. Pharmaceutical companies are naturally wary of marketing an antiviral which may affect the composition of bones, although there has been a suggestion that phosphonoformic acid may find use as an anti-herpes ointment for topical application. Our research into the mode of action of pyrophosphate analogues may lead to the development of other, active compounds which do not have unfortunate side effects.

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