ABSTRACT

A low molecular weight heparin (LMWH) obtained by a depolymerization process induced by a Fenton-type reagent was characterized in depth by nuclear magnetic resonance (NMR) spectroscopy. The depolymerization involves the cleavage of glycosidic bonds, leading to natural terminal reducing end residues, mainly represented by N-sulfated glucosamine. Natural uronic acids, especially the 2-O-sulfate iduronic acid, are also present as reducing residues. Unusual reaction results, such as the disappearance of the nonsulfated iduronic acid residues when followed by 6-O-nonsulfated glucosamine, and the decrease of the glucuronic acid when followed by the N-acetylg glucosamine, were observed. Iduronic acid residues, followed by 6-O-sulfate glucosamine (A$_{NS}$,6S), and the glucuronic acid residues, followed by A$_NS$ residues, were not modified. A few minor internal chain modifications occur, possibly arising from oxidative breaking of the bond between C2–C3 of glucosamine and uronic acids, suggested by evidence of formation of new –C=O groups. Finally, no change was observed in the content of the N-sulfated, 6-O-sulfated glucosamine bearing an extra sulfate on 3-O, which is considered the marker of the active site for antithrombin. With respect to the original heparin, this LMWH is characterized by a lower number of nonsulfated uronic acid residues, and as a consequence, by a lower degree of structural heterogeneity than LMWHs prepared with other procedures.

KEYWORDS:

Heparin 1 is a heterogeneous polysaccharide belonging to the family of glycosaminoglycans. It is constituted by disaccharide repeating units made up of uronic acid and glucosamine residues, each having different sulfation patterns. The possible different nature of the uronic acid (β-D-glucuronic acid [G], α-L-iduronic acid [I]), and their potential to bear a sulfate ester on carbon 2, combined with the different nature of the hexosamine residue (2-deoxy-2-sulfamino-α-D-glucose [A$_{NS}$], 2-acetamido-2-deoxy-α-D-glucose [A$_{NAC}$], or 2-deoxy-2-amino-α-D-glucose [A$_N$]), often O-sulfated at the primary carbon (A$_{6S}$) and sometimes at the carbon 3 (A$_{3S}$) or in both positions (A$_{3S,6S}$), allows a possible theoretical combination of up to 48 different disaccharides. In addition, the complexity of the system is increased by the presence of two different allowed equi-energetic conformations of the iduronic acid residues, responsible of the plasticity of some oligomeric heparin sequences, with respect to their target proteins. Even if not all of the disaccharide sequence permutations are allowed by the biosynthetic rules, the structural complexity of the polymer is high.

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The heparin chain is a macromolecule constituted mainly by two regions; the so-called N-acetylated (NA) and the N-sulfated (NS) domains. A NA–NS region is usually present between the two domains. Whereas N-sulfation gaps are confined prevalently in NA regions, the As–O-sulfation and I2–O-sulfation gaps are almost regularly spaced along the heparin chains. In each domain different permutations of the allowed disaccharides induce a large number of possible structural variants that account for the wide range of biological activities that heparin promotes by binding to different plasma and tissue proteins, such as protease inhibitors of blood coagulation cascade, growth factors, chemokines, adhesive matrix proteins, etc.7 The characterization of specific heparin binding structures responsible for the interaction with various proteins is of increasing interest. Although some proteins have affinity only for unique sequences in the heparin chain, others preferentially recognize the most regular regions of heparin, although this fact does not exclude selectivity of binding.8

Regular regions containing I2S and ANS,6S residues often are involved in nonanticoagulant activities of heparin and low molecular weight heparins (LMWHs). More specifically, these sequences have been recognized as those containing the binding sites for fibroblast growth factors (FGFs).10,9–13 A tetrasaccharide having such regular structure has affinity to FGF1 of about one order of magnitude higher than that of a corresponding tetramer, which lacks the 6-O-SO3 group on the non-reducing end of glucosamine.12 The opposite selectivity was observed for FGF2.14 Conversely, the structure depleted of both the 6-O-sulfates showed negligible activities.15 It was shown that binding and activation of FGF2 can be modulated by inducing sulfation gaps along the heparin chains, more specifically by removal of sulfate groups from I2S residues, followed by the splitting of C(2)–C(3) bonds of both the new and the original nonsulfated uronic acids (G and I). Notably, the observation that both partial 2-O-desulfation and the presence of flexible joints (glycol-split residues) between the unmodified heparin sequences are required for the inhibition of FGF2 activity and a potent antiangiogenic capacity of that heparin derivative.16

The anticoagulant activity of heparin is largely associated with interaction between a specific pentasaccharic sequence, ANA,6S–G–ANS,6S,ANS,6S–I2S–ANS,6S (AGA*IA), and antithrombin III (AT).17–19 Undersulfation may affect the anticoagulant/antithrombotic activity of heparin dramatically when it occurs at the level of essential SO3 groups of the active site for AT. The location of the AT binding region along the heparin chains is still not well defined. However, no ANS,3,6S residue has been found in heparin oligosaccharides containing the linkage region (the residual sequences located at the reducing end of the polysaccharide chain and joining it to the core protein of the original proteoglycan).20,21 This suggests that AGA*IA sequence should be located far from the reducing-end portion of the chain.

Because of different reasons, not all of the heparin preparations are structurally and biologically equivalent; one such reason is the origin of the sample. In fact, heparins of different origins have different patterns of sulfation and somewhat different contents of the active sequence for AT. Notably, some heparins extracted from clams are unusually rich in these sequences and display very potent anticoagulant properties.22 The structure of heparin depends not only on the animal species, but also on the original organs from which it was extracted. In the case of commercial preparations, the extraction procedure also can play a role on selection of more or less charged molecular species.23–25

Heparin is widely used in clinics as an anticoagulant. Since the 1980s, LMWH has been proposed as an antithrombotic drug. Different physical, chemical, or enzymatic procedures were developed to produce LMWH, thus generating variety of oligosaccharide components. In fact, each depolymerization approach is characterized by its own specificity, regarding both the point of cleavage (i.e., the preferred O-glycosidic bond that is cleaved), and the different chemical evolution of the new terminal residues.26–28 Such depolymerization mechanisms modify the amount of monomeric components and the number of permitted sequences that are responsible of different biological activities. The probability of preserving one specific oligosaccharide sequence is a function of the chain length, and the sequence activity is also function of its location inside the chain. For instance, the formation of a thrombin–antithrombin complex (i.e., expression of full anticoagulant activity) depends on the availability of six to seven residues of I2S–ANS,6S located at the nonreducing terminal side of the specific AGA*IA pentasaccharide sequence. Thus, for each AT binding sequence longer than AGA*IA, interactions with the AT could differ and give to each heparin fragment a specific biological response.29

**FENTON-TYPE DEPOLYMERIZATION REACTION**

In the present work, we studied the depolymerization of heparin with a Fenton-type reaction. In Fenton-type reactions, hydrogen peroxide is decomposed in a water solution in the presence of catalytic amount of a transition metal of a low oxidation number.30 The stoichiometry of the reaction is reported in the equation

\[
2\text{Me}^{n+} + \text{HOOH} + 2\text{H}^+ \rightarrow 2\text{Me}^{(n+1)+} + 2\text{H}_2\text{O}
\]

Typically, iron(II), but also other metal ions such as copper(II), inducing hydrogen peroxide decomposition,
can be involved in the Fenton-type reaction. As shown in Fig. 1, both of the processes are similar and are actually very complex, due to the high numbers of involved species and intermediate reactions. In addition, at present there is a debate about the real mechanism. Nevertheless, our discussions are based on the assumption that hydroxyl radicals are generated in some manner.

Hydroxyl radicals are very reactive and unselective in abstracting hydrogen atoms from a large spectrum of R-Hs, as shown in equation 1. The hydroxyl radical is strongly electrophilic and easily abstracts hydrogen from alcohols, ethers, and amides, especially from the carbons in oxygen or nitrogens. Accordingly, heparin can be involved in its action, with generation of several heparin radicals. The nature of R-H influences the overall system hydrogen peroxide/metal, given that R-CHO reacts with metal ions. Alpha-substituted radicals C(X = OH, OR, N) are usually oxidized both by Cu^{2+} and Fe^{3+} (the redox potential of alpha-hydroxy alkyl radicals is approximately ~1.18 V).

**Hydrogen Abstraction Generating Carbon-Centered Radical**

\[ \text{Me}^{5+} + \text{HOOH} \rightarrow \text{Me}^{(n+1)+} + \text{OH}^- + \text{OH}\bullet \]

\[ \text{OH}^- + \text{R}^- \rightarrow \text{H}_2\text{O} + \text{R}\bullet \quad (1) \]

The heparin used for the present study is a typical preparation from pig mucosal tissues with ~70% of its uronic acid residues as I_{2S}, and the remaining ones as I and G acids, in almost the same amounts. Its degree of sulfation is higher than 80% for both position C6-O and C2-N of glucosamine. The depolymerized reaction was performed in acidic medium (pH 4.5) at 70°C with 30% H$_2$O$_2$ for 6 hours, in the presence of Fe(II) sulfate. Then, the LMWH was precipitated with ethanol. The precipitate dissolved in water was desalted by Chelex resin to totally eliminate the catalyst. The obtained LMWH has a molecular weight (Mw) of 4200 d. To identify reducing groups, part of the sample has been reduced with NaBH$_4$. The samples have been characterized by nuclear magnetic resonance (NMR) spectroscopy.

**NMR Study of the LMWH**

$^1$H and $^{13}$C NMR spectroscopy are usually used for structural characterization of glycosaminoglycans. However, due to their high heterogeneity, LMWHs show complex monodimensional (1D) spectra, characterized by severe signal overlapping that does not permit reliable quantitative measurements. With proper choice of the analytical signals, the higher signal dispersions achieved with two-dimensional (2D) heteronuclear NMR methods also could be exploited for quantitative applications.

The $^1$H/$^{13}$C heteronuclear single quantum coherence (HSQC) NMR spectrum of the LMWH obtained by the Fenton depolymerization is shown in Figs. 2A and 2B. The $^1$H and $^{13}$C monodimensional spectra, displayed respectively on F2 and F1 HSQC spectral axes, reveal that the sample maintains a typical heparin profile. Conversely, the 2D spectrum shows the presence of minor signals overlapping those commonly integrated to determine the sulfation pattern by 1D spectra. To obtain a more detailed picture of the monosaccharide composition of the LMW products, and to avoid errors due to signals overlap, the determination of sulfation pattern was done by integration of more resolved HSQC cross-peaks.

Table 1 summarizes the quantitative composition of a LMWH sample representative of this specific depolymerization procedure. The NMR data are correlated with the Mw values obtained by gel permeation chromatography measurements. The depolymerized sample shows the expected Mw reduction, with respect to the parent heparin. NMR signals of reducing end ANS and I$_{2S}$ residues were quantified. The A$_{NSC}$ is the prevalent terminal residue. Indeed, the 2D spectrum shows the presence of other reducing end signals, such as A$_{NAS}$, I, and G, but their intensities are too low to permit quantification. This limitation is justified by the low sensitivity of the current NMR instrument and by the dispersion of reducing-end proton signals. Such
Figure 2  (A) Total $^1$H, $^{13}$C nuclear magnetic resonance (600 MHz) heteronuclear correlation spectrum of low molecular weight heparin. The C4 signals of the nonreducing end N-sulfated D-glucosamine ($\text{ANS}_{2}$) and D-glucuronic acid (G) are clearly detectable. (B) Expansion of the anomeric region: intensities of the D-glucosamine (unknown structure; $\text{AX}$) signal and of the N-acetyl D-glucosamine ($\text{ANAc}$) signal are increased by a factor of two. $\text{r}$, reducing; $\text{ANS}_{3S,6S}$, N,3-O-disulfated D-glucosamine; $\text{ANS}_{3S,6OH}$, ???; $\text{ANAc}_{3S,6OH}$, ???
a dispersion occurs because of differences in chain sulfation pattern, sequence effects, chemical type of the end residue, and α and β configurations. The content of the total N-sulfated glucosamine (ANS) as well as of the marker of the active site for antithrombin (ANS,3,6S) correspond to that observed in the original heparin. Under stronger reaction conditions, the ANAc content is slightly different with respect to that of heparin, and the content of non sulfated iduronic acid (I) decreases with respect to the total uronic acid (U) residues.

The HSQC spectrum of heparin can be analyzed in a quantitative way, not only in terms of monosaccharide units, but also in terms of dimer sequences. The molar relative percentages distribution of the detectable dimeric sequences are reported in Tables 2 and 3 for uronic acid and hexosamine residues, respectively.

The 1D spectrum reported in Fig. 3 shows the anomeric region of the proton spectra of the parent heparin, the LMWH, and its reduced derivative. As shown by the 2D HSQC spectrum (Fig. 2A), in the LMWH spectrum the signal at 4.94 ppm of the anomeric residue I followed by A6OH (IQ19-ANS,6OH) disappears, whereas residual minor signals due to nonanomeric resonances are present. These signals are assigned to H5 proton of I2S and are associated with a higher structural complexity of the LMWH with respect to heparin. The profile of the spectrum of the reduced derivative shows a change. In particular, the most downfield signals disappear.

The HSQC spectrum of the present LMWH is characterized by some minor new signals, the intensity of which depends on the conditions of the depolymerization reaction. Among them, in the high-field spectral region (Fig. 2A), C4/H4 correlation signals of the nonreducing terminal residues of both ANS and G are well evident in a relative ratio of 2:1. The ANSr is the 5-molar percentage of the total glucosamine.

In the anomeric spectral region (Fig. 2B), different sets of minor signals are present. Two of them are detected at 5.35/100.1 and 5.23/103.3 ppm. A shift of the resonance of the last signal (5.29/102.8 ppm) is induced by NaBH4 treatment, suggesting that this signal might be attributed to a moiety linked to a reducing end-terminal residue, whereas the unmodified 5.35/100.1 signal belongs to H1/C1 of ANS,6OH linked to an I2S. Other signals are present at a lower field (Ax; 5.73 and 5.71 ppm in 1H axis [F2] and 103.0 ppm in the 13C axis [F1]). Analysis of a sample obtained under more drastic reaction conditions indicates that each of these last signals in the 2D correlation spectroscopy and in the 2D total correlation spectroscopy spectrum corresponds to only one correlation peak, at 4.48 and 4.43 ppm respectively, corresponding to 65.4 ppm in 13C spectrum; as a consequence, these two signals belong to two modified rings. For the 5.73 ppm signal, which belongs to one of the modified rings, the 2D NOESY experiment shows its dipolar correlation with C4 of both the I2S and G previous residues (Fig. 4) at 4.23 and 4.17 ppm, respectively. This last result suggests that this modified ring comes from a glucosamine residue, which can be linked either to a glucuronic or to a 2-O-sulfated iduronic acid residue.
Regarding the second one, it is impossible to observe its dipolar correlation signals because they are too weak. For both of the modified rings, the absence of correlation peaks of C2 with a C3 and with additional carbons is noteworthy, suggesting a possible ring opening.

In the 2D heteronuclear multiple bond correlation experiment (HMBC), it is possible to follow hydrogen–carbon correlations through a three-bond distance. This experiment is especially useful for the observation of carbonyl correlations. Fig. 5 shows the partial spectrum relative to carbonyl correlations of the LMWH. At field higher then 177 ppm (F1), at least five new 13C correlations with 1H signals appear in the spectrum in the area between 3.9 and 5 ppm (F2). Between them, one (178.5 ppm) is interconnected with the methyl of a

Figure 3  1H nuclear magnetic resonance (500 MHz) complex anomeric signals of (B) low molecular weight heparin (LMWH) compared with (A) parent heparin, and (C) LMWH after NaBH4 treatment. I2S, 1-iduronic acid 2-O-sulfate; ANSE3S, N,6-O-disulfated D-glucosamine; ANX, D-glucosamine unknown structure; G, D-glucuronic acid; ANS,3S, N,3-O-disulfated D-glucosamine; ANS,5OH, ???Q20.

Q22

STRICTURAL MODIFICATION INDUCED IN HEPARIN/ VISMARA ET AL

Figure 4 Partial 1H, 13C nuclear magnetic resonance (600 MHz) heteronuclear multiple bond correlation of low molecular weight heparin showing the correlation peaks of –COR signals with protons at two-three-bond distance.
N-Ac glucosamine, one belongs to a new I5 2S, and another one (179.2 ppm) corresponds to the previously described signal of the C2 at 4.43 ppm.

DISCUSSION

The mechanism of hydroxyl radical action on heparin can be discussed starting from NMR results. If we consider the common behavior of a hydroxyl radical, which is highly reactive and unselective (as discussed), all of the heparin hydrogen atoms could be abstracted, in principle. However, if we consider the heterogeneous polymeric nature of heparin and also the different charged functional groups, it is not difficult to imagine that not all of the hydrogen atoms are equivalent toward hydroxyl radical action.

In Fig. 7, the second mechanism passes through anomeric hydrogen atom abstraction followed by oxidation of the anomeric radical to a carboxylation responsible for the glycosidic bond breaking, which can lead finally to an aldonic acid terminal formation. This mechanism justifies the formation of the observed natural nonreducing end terminals, and can be responsible in part for the observed carbonyl and carboxyl signals on HMBC spectrum.

In Fig. 8, we propose the third mechanism, which, as in the first mechanism, starts with primary radical formation from C2 and C3, both on uronic or on glucosamine rings. In this case, a subsequent oxidative pathway occurs, which passes through oxygen trapping by
the primary radical to form a peroxide evolving in a ring opening (C2–C3 bond breaking), followed by an oxidation of the C radical to new carbonyl and/or carboxyl functional groups. To follow such a mechanism, we can refer to the well-known autoxidation processes involving oxygen and radical intermediates. In addition, this mechanism can be responsible in part for the observed carbonyl and carboxyl signals on the HMBC spectrum. Notably, preliminary mass analyses have identified oligosaccharides ending with the proposed remnant shown in Fig. 8.

The total recovery of glucosamine is not affected by the reactions whose β scission leads to the glycosidic bond breaking, generating a reducing or nonreducing end terminal, respectively. This is in agreement with the presence of a 5 to 6 molar percent concentration of both ANS reducing and nonreducing end-terminal residue (ANSr, ANSnr). Table 2 shows the total recovery
of aminosugar, divided into reducing and internal residues; the reducing end residues mainly come from breaking of disaccharides containing I\textsubscript{2S} or I. This pathway influences 6% of the starting aminosugar. Notably, measurement of the nonreducing end ANS\textsubscript{nr} can help to quantify the total amount of uronic reducing terminals that are usually highly dispersed. In fact, Table 1 reports the molar content (2%) only for the α component of the I\textsubscript{2S} signal, whereas the other uronic residues are not quantifiable or detectable. Conversely, the ANS\textsubscript{nr} signal is not affected either by different configurations or by sequence effects, given that its corresponding proton is located at the other side of the glycosidic bond. This last observation can help to evaluate the degree of depolymerization via NMR technique.

For glucosamine residues, the previously described oxygen trapping by the primary radical, and the subsequent reactions, are suggested by the interruption of the correlation signal sequences in the NMR 2D total correlation spectroscopy [27] spectrum and by the spatial correlations generated by the anomeric minor signals at 5.73 and 5.71 ppm to H4 of I\textsubscript{2S} and G residues (Fig. 5). Furthermore, as reported previously, Fig. 3 shows the disappearance (shift) of this anomeric signal when the sample is reduced by NaBH\textsubscript{4}.

From Table 3, it appears that the glycosidic bond of iduronic acid in the sequence I\textsubscript{o} ANS\textsubscript{6,5} seems practically unaffected. In contrast, the unsulfated glucuronic residue seems partially involved in the depolymerization reaction only when it is followed by an N-acetyl glucosamine (G\textsubscript{o} ANAc). In this last case, the NMR spectra do not allow one to distinguish between hexosamine sequences bearing C6-OS or C6-OH. However according to biosynthetic rules,\textsuperscript{9,17,18} it is known that both sequences are present and the nonsulfated dimer is likely the most represented sequence. The observation of the decreasing content of G\textsubscript{o} ANAc was made only through the disaccharide sequence analysis, whereas this analysis is based on quantification of the only signals that are attributable.

Regarding uronic acids, the decrease of molar percent of nonsulfated uronic acid moieties in the depolymerized indicates that they are also reaction targets. However, the expected amount of regular unsulfated reducing terminal was not found. The disappearance of these residues could be explained by the previously described second mechanism, which comprises several minor side reactions. In fact, the heteronuclear long-range correlation (HMBC) NMR experiment shows at least four new carbonyl functional groups interacting through a three-bond distance with hydrogen of secondary and primary alcohols and methyl residues.
CONCLUSION
The LMWH preparations obtained using a Fenton depolymerization process through iron ions catalysis are characterized by natural reducing and nonreducing end-terminal residues generated by cleavage prevalently at the level of glucosamines. This cleavage introduces in the sample both even and odd chains of oligomers. The amount of disaccharides containing an unsulfated uronic acid is reduced significantly. In particular, in the depolymerized product the sequence 1-ANSOH is practically absent, due to both the proposed mechanisms, one of which allows the formation of natural terminals, the other that induces at least four oxidized and/or ring-opened derivatives, generated by minor

Figure 8 Oxidative ring opening and remnant formation.
side reactions. Among these side reactions, the lack of such uronic rings and formation of remnant cannot be excluded. As a consequence, the depolymerization process preserves in the heparin chains the most sulfated disaccharide units, mainly corresponding to the trisulfated disaccharide I$_{2S}$-A$_{NS}$,6S, that are prevalent in the NS domains. The decrease of the nonsulfated iduronic acid, which probably is randomly distributed in the heparin chain, suggests the formation of more homogeneous oligomeric sequences, coming from the heparin NS regions. The quantitative recovery of A$_{NS,3S,6S}$ residues and of the glucuronic acid followed by A$_{NS,3S,6S}$ residues, suggests that the active site for AT is largely preserved in the present LMWH.

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ABBREVIATIONS

1D monodimensional
2D two dimensional
A$_{3S}$ 3-O-sulfated D-glucosamine
A$_{6S}$ 6-O-sulfated D-glucosamine
AGA*IA pentasaccharide A$_{NAc,6S}$–G–A$_{NS,3,6S}$–I$_{2S}$–A$_{NS,6S}$
A$_{N}$ d-glucosamine
A$_{NAc}$ N-acetyl d-glucosamine
A$_{NS}$ N-sulfated D-glucosamine
A$_{NS,3S}$ N,3-O-disulfated d-glucosamine
A$_{NS,6S}$ N,6-O-disulfated d-glucosamine
A$_{NS,6S,3S}$ N,6-O,3-O-trisulfated d-glucosamine
A$_{NSr}$ nonreducing end N-sulfated d-glucosamine
A$_{NSr}$ reducing end N-sulfated d-glucosamine
AT antithrombin III
A$_{X}$ d-glucosamine unknown structure
d$_{2}$O deuterium oxide
FGF fibroblast growth factor
G d-glucuronic acid
GAG(s) glycosaminoglycan(s)
HMBC 2D heteronuclear multiple bond correlation
HSQC 2D heteronuclear single quantum coherence
I L-iduronic acid
I$_{2S}$ L-iduronic acid 2-O-sulfate
LMWH low molecular weight heparin
NA N-acetylated heparin domain
NMR nuclear magnetic resonance
NS N-sulfated heparin domain
TSP trimethylsilyl propionate sodium salt

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<td>3rd Edition</td>
<td>Professor of Pathology, University of British Columbia; Pathologist, Vancouver Hospital &amp; Health Sciences Center, Vancouver, BC, Canada</td>
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<td>Cerebral and Peripheral Vessels</td>
<td>Professor and Chairman, Department of Neurology, University of Heidelberg, Mannheim, Germany</td>
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Covering the entire venous and body circulation as examined by vascular ultrasound, this unique text/atlas is invaluable for diagnosing arterial and venous disease. It includes comprehensive chapters on vascular ultrasonography in the arteries and veins of the cerebral circulation and the peripheral upper and lower limb circulation, systematic coverage of all available ultrasound technologies, including continuous and pulsed-wave Doppler mode, b-mode, and conventional and color-coded duplex analysis in frequency and amplitude power modes, anatomy and physiology, normal and abnormal findings, test accuracy and sensitivity, pitfalls, and comparison with other diagnostic tests in each vascular region and special, difficult-to-interpret cases discussed in a separate section.

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