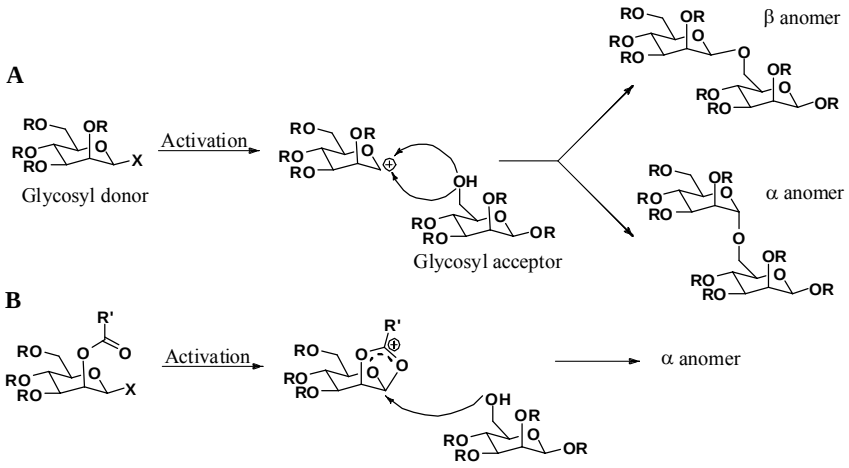
AUTOMATED SOLID-PHASE SYNTHESIS OF OLIGOSACCHARIDES

By Daniel Palacios April 23, 2007

INTRODUCTION

Three main classes of biopolymers are present within the human body: polypeptides, oligonucleotides and oligosaccharides. Of the three, understanding of the rich and varied roles played by oligosaccharides in living systems remains the most incomplete.[1] Reliable access to pure oligosaccharides in quantities suitable for biophysical studies remains a major barrier to our growing knowledge of these fascinating biomolecules. Oligosaccharides are present in very low concentrations in biological systems, making their isolation from natural sources difficult and laborious. Therefore, the production of oligosaccharides is entrusted to a small community of highly specialized synthetic organic chemists in contrast to polypeptides and oligonucleotides, the syntheses of which are routine and usually automated. The need for a simple, dependable method for the synthesis of highly complex oligosaccharides by non-specialist would therefore revolutionize our understanding of complex oligosachharides.

Scheme 1. A general glycosidation reaction



As shown by Merrifield[2] and Caruthers,[3] solid-phase synthesis is the optimal approach for the automated synthesis of biopolymers. Several key challenges must be addressed, however, when attempting the development of an automated solid-phase synthesis. First, the yield of each coupling step must be high, preferably greater than 95%, because synthetic intermediates cannot be purified. A key challenge for oligosaccharides, which is not an issue for polypeptides or oligonucleotides, is that every coupling step must also proceed with excellent diastereoselectivity because each glycosidation may produce either an α or β anomer (Scheme 1A). The selectivity issued may often be solved through judicious selection of protecting groups as several classes of protecting groups, notably esters, are able to influence diastereoselectivity through neighboring group participation (Scheme 1B). In addition, both the solid-phase linker and the solid-phase support must be inert to all the reagents present in the coupling cycle

yet must also be cleanly removed at the end of the synthesis without causing deleterious side reactions. Finally, care must be taken in the protecting group strategy such that reactive groups may be selectively deprotected and coupled without disturbing other sensitive functionality present within the molecule.

Automated synthesis of a polymannoside

The initial demonstration of solid-phase peptide synthesis in 1963 by Merrifield[4] inspired Fréchet to undertake the first attempt of a solid-phase oligosaccharide synthesis in 1971.[5] Despite this initial success, solid-phase oligosaccharide synthesis was dormant for approximately 20 years due to the lack of reliable glycosylating reagents and powerful analytical tools for on-resin analysis. During this time, however, many advances were made in the area of solution-phase oligosaccharide synthesis including the development of highly selective glycosyl donors as well as a diverse set of orthogonal protecting groups. In addition, incredibly powerful tools such as high-resolution solid-state nuclear magnetic resonance slowly evolved and are now routine. These advances led to renewed interest in solid-phase oligosaccharide synthesis,[6] generating a large body of literature on the topic.[7]

This accumulated body of literature enabled Seeberger and coworkers to confront the issues outlined in the introduction and attempt the first automated solid-phase synthesis of an oligosaccharide.[8] As mentioned above, the choice of solid-phase linker and support is critical to the success of any solid-phase synthesis. The linker chosen was olefin **3** (Scheme 2), which is stable to both acidic and basic conditions but is readily cleaved in high yield by olefin metathesis under an atmosphere of ethylene. Furthermore, the resulting *n*-pentenyl glycoside can serve as a glycosyl donor, or it may be selectively deprotected by aqueous *N*-bromosuccinimide.[9] For the polymeric support, Merrifield resin was empirically chosen based on previous manual solid-phase syntheses.

The initial targets for automated synthesis were a series of α-(1→2) mannosides, (Scheme 2, structures **5-7**) becauseoligosaccharides of this type had been previously synthesized both on the solid-phase and in solution[8] and thus provided a basis for comparison. The synthesis began with the preparation of the solid support. Diol **1** was protected as a dimethoxytrityl (DMT) ether and then loaded onto Merrifield resin. Deprotection of the DMT group with dichloroacetic acid allowed for the colormetric quantification of the amount of linker covalently bound to the solid support. Resin loadings were in the range of 0.1-0.3 mmol/g resin, indicating this method is suitable for the synthesis of compounds in sufficient quantity for biophysical and biological examination. Olefin **3** was then coupled with the trichloroacetimidate glycosyl donor **4** to initiate the automated synthesis. Mannoside **4** was chosen as the glycoside donor for this iterative synthesis because it is rapidly prepared on a multi-gram scale, bears a C2 acetate for control of stereochemistry at the forming anomeric position, and is activated

Scheme 2. Automated synthesis of polymannosides

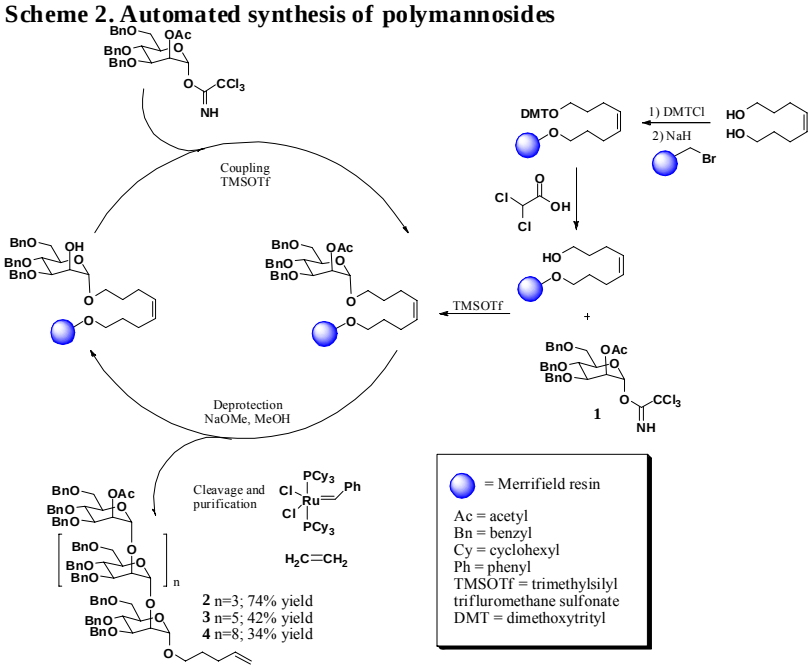


Table 1. Trichloroacetimidate coupling/deprotection cycle

Step	Function	Reagent	Time (min)
1	Couple	10 eq. donor and 0.5 eq TMSOTf	30
2	Wash	CH_2Cl_2	6
3	Couple	10 eq. donor and 0.5 eq TMSOTf	30
4	Wash	CH_2Cl_2	6
5	Wash	1:9 MeOH:CH_2Cl_2	6
6	Deprotection	10 eq. NaOMe in 1:9 methanol:CH_2Cl_2	30
7	Deprotection	10 eq. NaOMe in 1:9 methanol:CH_2Cl_2	30
8	Wash	1:9 MeOH:CH_2Cl_2	4
9	Wash	0.2 M acetic acid in tetrahydrofuran	4
10	Wash	tetrahydrofuran	4
11	Wash	CH_2Cl_2	6

at room temperature. Deprotection of the 2-*O* acetate with sodium methoxide in a mixture of methanol and dichloromethane then generated the glycosyl acceptor on the resin. This acceptor-bound strategy was chosen over the donor-bound because glycosyl donors are prone to side reactions, adversely affecting the yield. To avoid deletion sequences, which can complicate purification of the final compound, glycosidation and deprotection steps were performed twice as shown in Table 1. Using this iterative cycle allowed the construction of heptamer **6** in 42% overall yield in merely 20 hours with each coupling step averaging 90 to 95% yield. In comparison, the same heptamannoside was synthesized manually on Merrifield resin in 14 days and 9% overall yield.[9]

Automated synthesis of a branched oligosaccharide

The remarkable success of this initial attempt as well as its striking improvement over manual solid-phase synthesis prompted Seeberger and coworkers to attempt the automated synthesis of a more complex structure, the fungal phytoalexin elicitor β-glucan **10** (Scheme 2), which induces soybean plants to secrete antifungal phytoalexins.[11] This more complex structure required careful planning of protecting group strategy to ensure the 2-*O* directing group is not unmasked when the 6-*O* hydroxyl is generated for the coupling step. To ensure protecting group orthogonality, Seeberger and coworkers chose a levulinoyl ester for the temporary 6-*O* protecting group because it is readily deprotected with hydrazine, while a 2-*O* pivolate ester was installed to ensure high levels of β selectivity. Previously unknown glycosyl phosphates **8** and **9** were chosen as glycal donors due to the superlative performance

Scheme 3. Automated synthesis of a hexameric β-glucan phytoalexin elicitor

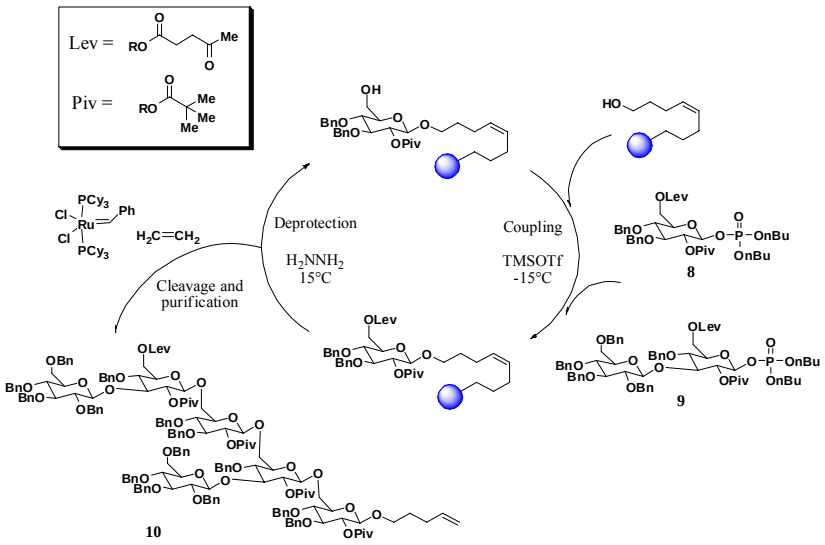


Table 2. Glycosyl phosphate coupling/deprotection cycle

Step	Function	Reagent	Time (min)
1	Couple	5 eq. donor and 5 eq. TMSOTf	30
2	Wash	CH_2Cl_2	6
3	Couple	5 eq. donor and 5 eq. TMSOTf	30
4	Wash	1:9 MeOH:CH_2Cl_2	4
5	Wash	tetrahydrofuran	4
6	Wash	3:2 pyridine:acetic acid	4
7	Deprotection	20 eq. hydrazine in 3:2 pyridine:acetic acid	30
8	Deprotection	20 eq. hydrazine in 3:2 pyridine:acetic acid	30
9	Wash	3:2 pyridine:acetic acid	4
10	Wash	1:9 MeOH:CH_2Cl_2	4
11	Wash	0.2 M acetic acid in tetrahydrofuran	4
12	Wash	tetrahydrofuran	4
13	Wash	CH_2Cl_2	6

of glycosyl phosphate donors in both solution and solid-phase syntheses.[12] However, glycosyl phosphates, among other glycosyl donors such as glycosyl sulfoxides and *N*-phenyl trifluoroacetimidates, require sub-ambient temperatures in order to couple effectively. This provided an additional challenge for the synthesis, in addition to offering a chance to demonstrate the robust nature of this technology. The construction of a temperature controlled reaction vessel was therefore required because the automated synthesis of polypeptides and oligonucleotides are performed completely at room temperature. This problem was readily overcome by enclosing the reaction vessel within a cooling jacket attached to a commercially available cryogenic cooler, allowing temperature variation to be incorporated into the reaction cycle.

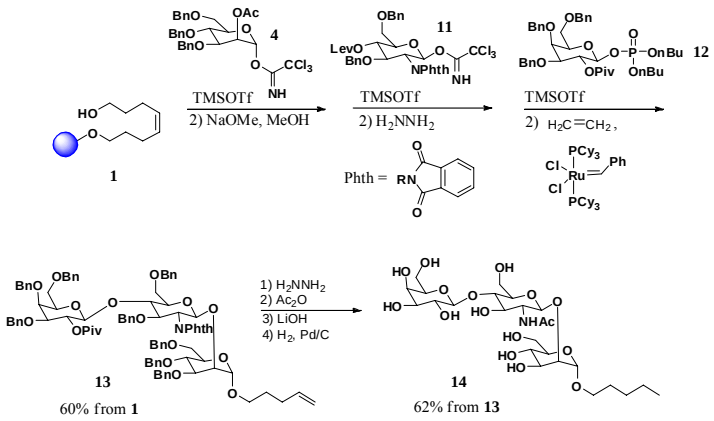
Based upon model studies in solution, a thirteen-step coupling cycle was constructed (Table 2). The glycosyl donor, either **8** or **9**, was activated with trimethylsilyl trifluoromethanesulfonate (TMSOTf) at -15 °C and was coupled to the solid-bound glycosyl acceptor. Deprotection of the 6-*O* levulinoyl group with hydrazine proceeded smoothly at +15 °C generating the glycosyl acceptor for the next coupling. Again, two cycles of coupling and deprotection were employed to ensure complete reactions and an additional wash step was also used, 3:2 pyridine:acetic acid for 4 minutes, to make certain that all of the hydrazine was removed. Thus, by appropriate use of either glycal donor **8** or **9** in the coupling step, the branched oligosaccharide **10** was rapidly assembled in only 10 hours and in >80% yield as determined by HPLC, with each cycle (coupling and deprotection) proceeding in approximately 95% yield. In addition to the hexamer **10** shown, a dodecasaccharide was also synthesized in 17 hours and in

>50% yield using the same cycle. Although this synthesis demonstrated that branched oligosaccharides are within the scope of this technology, the synthesis failed to incorporate the actual branching event. Instead, branched building blocks were used, requiring the specialized knowledge and skill necessary for the synthesis of a disaccharide. It is conceivable, however, that future generations of this technology could incorporate the branching event into the automated process through the use of orthogonal protecting groups.

Incorporating glucosamine

Having demonstrated the feasibility of automated solid-phase oligosaccharide synthesis, Seeberger and coworkers then sought to illustrate the generality of this method by synthesizing trisaccharide **13** (Scheme 4), which incorporates all the features of this automated chemistry established by the previous two syntheses. Structures containing this trisaccharide composition present a significant synthetic challenge as the installation of glucosamine the C2 position of mannose is notoriously difficult.[13] Careful deliberation was therefore required in choosing the appropriate glycal donors for the synthesis of **13**. The initial donor **4** was chosen due to its previous performance in the synthesis of the polymannoside **6**. Glucosamine donor **11** was fashioned to incorporate lessons learned in the synthesis of **10** by protecting the 4-*O* functionality with a levulinoyl ester because of its demonstrated ease of deprotection. A 2-*N* pthalimide protected amine was installed in order to confer high β selectivity in the glycosidation step. Glycosyl phosphate **12** was chosen as the final coupling partner because these glycosyl donors are known to be very reactive and thus suitable for constructing sterically demanding glycosidic bonds.[12]

Scheme 4. Automated synthesis of a trisaccharide



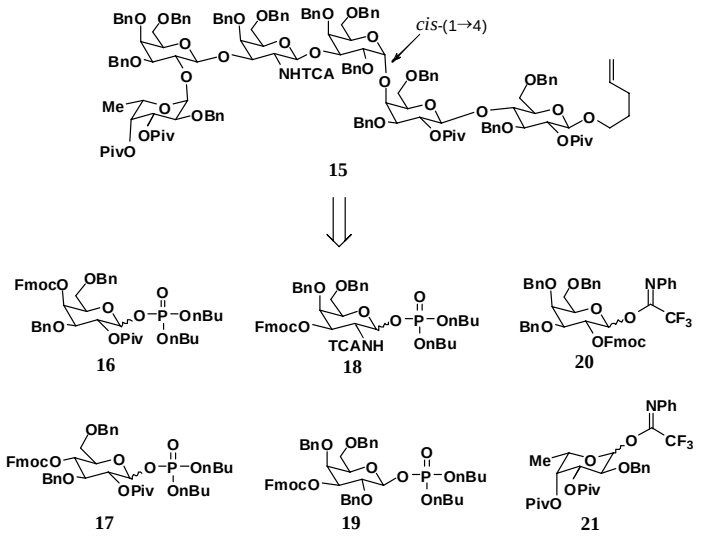
The automated solid-phase synthesis of **13** began with the attachment of trichloroacetimidate donor **4** to Merrifield resin using the coupling/deprotection cycle shown in Table 1. The temperature for the coupling of **11** to the 2-*O* mannoside hydroxyl was optimized to –15 °C and this was followed by the levulinoyl removal conditions outlined in Table 2. The free 4-*O* hydroxyl was then coupled to glycosyl donor **12** using the conditions developed for the synthesis of **10**. The solid-bound trisaccharide was subsequently released from the solid support using Grubbs' first-generation catalyst under an atmosphere of ethylene, generating fully protected **13** in 60% yield from alcohol **1** with an average

coupling/deprotection sequence of 85%. This yield is significantly lower than the previous syntheses described above, which averaged 95% per step. The lack of an on-resin assay for the evaluation of reaction success, it is unknown which glycosidation proved problematic and led to the decreased average yield. To establish that oligosaccharides synthesized in this manner can be readily deprotected, trisaccharide **13** was taken forward to **14** using standard deprotection protocols in 62% yield over 4 steps.

Synthesis of tumor-associated carbohydrate antigens

Albeit groundbreaking, the work reported by Seeberger and coworkers[8] examined a mere subset of the myriad of glycoside connections found in nature. Among these unexplored linkages was a *cis*-(1→4) galactosidic linkage. The stereochemical outcome of this glycosidation is infamously immune to stereochemical control via neighboring esters. In addition to this formidable glycoside coupling, another outstanding problem which was not previously addressed was the inability to assess the effectiveness of each coupling step without cleaving the growing oligosaccharide from the solid support. In an attempt to address these problems, Seeberger and coworkers targeted the protected glycosphingolipid Globo-H (**15** Figure 1) for synthesis.[14] Globo-H is expressed on the surface of several types of cancers and is currently being evaluated in clinical trials as a vaccine for breast and prostate cancers.[15] Hexasaccharide **15** is broken down into six protected monosaccharides **16-21**, five of which contain a temporary protecting group. Fucose moiety **21** is the exception since it is the final monosaccharide installed.

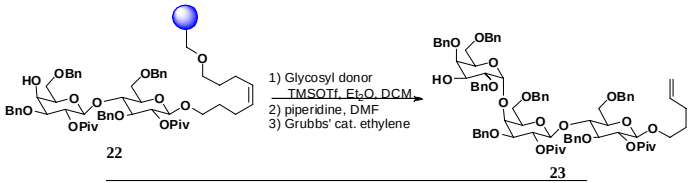
Figure 1. Tumor-associated antigen Globo-H and its building blocks



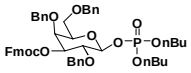
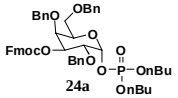
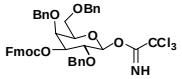
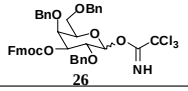
The temporary protecting group chosen for this automated solid-phase synthesis was fluorenylmethoxycarbonyl (Fmoc), which it is stable to the acidic coupling conditions and is cleaved with a mild amine base such as piperidine. In addition, Fmoc has a proven track record in automated peptide synthesis because analysis of the solution containing the deprotected Fmoc provides a quantitative colormetric assay for the efficiency of each coupling/deprotection step. This makes troublesome couplings readily identifiable. The additional level of protecting group orthogonality also

provides an opportunity to incorporate branching into the automated synthesis, which was not possible in the first iteration of this technology (see above). Before attempting the synthesis of **15**, however, construction of the *cis*-(1→4) galactosidic linkage was investigated using various glycosyl donors, the results are summarized in Scheme 5. High diastereoselectivity for this reaction are crucial to the success of the synthesis because there is no opportunity to purify any of the intermediates until cleavage from the solid support. The glycosidations were performed with an automated solid-phase synthesizer and the coupling efficiency and selectivities were evaluated by LC-MS analysis following cleavage from the resin. The conversion of **22** to **23** showed a conspicuous dependence upon the anomeric configuration of the glycosyl donor, as evidenced by entries 1 and 2. The β phosphate **24b** reacted with much higher diastereoselectivity in comparison to the α anomer **24a** (14:1 vs. 4:1 dr). Results were similar for the trichloroacetimidate donor.

Scheme 5. Optimizing formation of the *cis*-(1→4) linkage

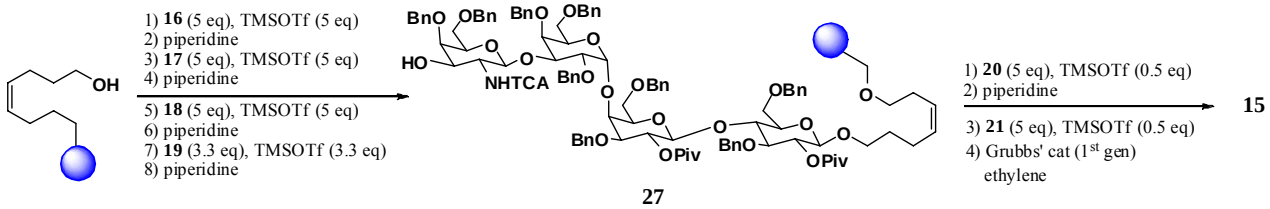


22 → 23

Entry	Glycosyl donor	α:β selectivity of **23**
1	 **24b**	14:1
2	 **24a**	4:1
3	 **25**	11:1
4	 **26**	4:1

Satisfied with the 14:1 diastereoselectivity, Seeberger and coworkers went forward with the synthesis of protected Globo-H **15** as shown in Scheme 6. During the course of the synthesis it was discovered that tetrasaccharide **27** was unstable to the stochiometric amounts of TMSOTf required for the activation of glycosyl phosphates. Therefore, the *N*-phenyl trifluoroacetimidate activating group was substituted for the final two couplings because it requires sub-stochiometric amounts of TMSOTf for activation. Following the final to glycosidic couplings the fully protected solid-bound Globo-H was exposed to Grubbs' catalyst under an atmosphere of ethylene to give **15**.

Scheme 6. Synthesis of protected Globo-H



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Conclusions and future directions

Though an area of study still in its infancy, the development of an automated oligosaccharide synthesizer has demonstrated the potential of becoming a powerful tool in the understanding of oligosaccharides. Syntheses that once required weeks can now be complete in hours and with a pronounced increase in yield relative to manual solution and solid-phase syntheses. Significant advances have been made since the introduction of this technology, such as the development of an on-resin assay for reactions, the synthesis of *cis*-(1→4) galactosidic linkages and incorporation of branching into the automated synthesis cycle. Considerable challenges remain unsolved, however. For instance, the most common donors used, glycosyl phosphates and trichloroacetimidates, are not shelf stable and require synthesis and purification immediately prior to use. The ideal donor would be sufficiently stable to be commercially available, yet reactive enough for sterically demanding coupling reactions. Also, naturally occurring oligosaccharides are most often found as conjugates with either proteins or lipids.[1] Despite this, no attempts[16] have been made to incorporate lipids or peptides into the automated cycle, which would require additional levels of protecting group orthogonality. The uncharted potential impact on human health afforded by synthetic oligosaccharides leaves no doubt, however, that a machine capable of rapidly synthesizing complex oligosaccharides remains a goal worth realizing.

References

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